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Penetration of Ultraviolet Radiation Into Skin, as a Factor in Carcinogenesis

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The proper interpretation of experiments dealing with induction of tumors by ultraviolet radiation requires knowledge of the penetration of this agent into skin. This is particularly important as regards types of tumor produced, since the amount of radiation penetrating to the different layers limits the extent to which the various cell types which make up those layers may be involved. It is of special interest when one considers the relationship of sunlight to human cutaneous cancer. Almost all such tumors are epidermal in origin, whereas those induced by ultraviolet radiation in albino mice arise chiefly in the dermis (*1*). The comparative studies of penetration of ultraviolet radiation into human and mouse skin, which are presented in this paper, were undertaken to assist in the solution of such problems.

EXPERIMENTAL PROCEDURE

Transmission of ultraviolet radiation differs for the various layers of which skin is composed, and accurate measurement of the radiation absorbed in each would require its separation and individual study. At present, methods for separating all the layers are not available, but, fortunately, it is possible to separate the epidermis from the underlying tissues, and to measure its transmission. The amount of radiation reaching the dermis is one of the more important questions which requires an answer, and this is susceptible to direct determination by measuring the transmission of samples of separated epidermis.

The epidermis is an inhomogeneous medium in which scattering as well as absorption [1] of radiation occurs. The scattering results principally from multiple reflection and refraction at surfaces of discontinuity rather than from molecular scattering. This is shown by the fact that the scattering is approximately independent of wavelength (*2*), whereas molecular scattering is inversely proportional to the fourth power of the wavelength. It is probable that the scattering occurs chiefly in the corneum. This layer is made up of scalelike elements which represent dead cells, and these present a series of surfaces at which reflection and refraction may occur. Interstices between these scalelike elements are probably filled with air or

[1] *The term "absorption" is here used to denote true absorption exclusive of diminution of intensity by scattering.*

water, for clearing may be accomplished by applying a substance with appropriate refractive index which fills these spaces, and hence reduces the reflection and refraction. The viable cells of the mucosum, on the other hand, probably act more nearly as a simple absorbing medium due to the greater optical homogeneity at the borders between the living cells and other surfaces within the cells at which reflection and refraction occur. At any rate, the radiation which reaches the mucosum is already diffused over a wide solid angle.

If, following the customary procedure of absorption spectrophotometry, a collimated beam is made to impinge upon a sample of epidermis, the radiation is immediately diffused, and that emergent portion which would be detected by a spectrograph placed in the usual manner so as to receive the incident collimated beam would represent a very small fraction of the transmitted radiation. Measurements carried out in this manner have yielded qualitative results (*3*, *4*).

Two optically equivalent methods are available for measurement of transmission by such an inhomogeneous medium. The epidermis may be illuminated with diffuse radiation and the emerging radiation measured with a spectrograph placed in the usual position (*5*, *6*, *7*). One difficulty with this method is that the radiation included in the narrow angle accepted by the spectrograph is so weak as to be very difficult to measure. In order to obtain measurements at shorter wavelengths where the transmission was low, Lucas (*5*) found it necessary to clear the epidermis with acetic acid in order that the transmitted radiation would be sufficient to measure. Furthermore, this method subjects the tissue to the action of the whole spectrum which may cause undue heating or other effects.

Another method was employed in the present investigation, the epidermis being illuminated with a collimated beam of monochromatic radiation, and the whole of the emerging radiation estimated photoelectrically.[2] This is essentially the method used by Bachem and Kunz (*8*), but important modifications were made.

Estimation of the total emerging radiation presented the principal difficulty of the method and was accomplished in two ways.

The first method is illustrated in figure 1. *S* is the slit of a large double monochromator (aperture *F* 4) by means of which the lines of a mercury arc source are isolated to provide monocromatic radiation of great purity. *L* is a short focus plano-convex lens placed in contact with the exit slit of the monochromator so as to form an image of the prism face 6 mm. square at the plane of the diaphragm *D*. The collecting lens system *C* is placed

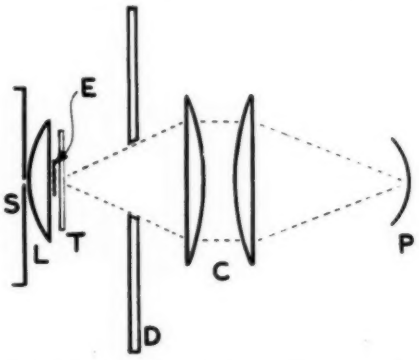

FIGURE 1.—Diagram of apparatus. *S*, exit slit of monochromator; L, plano-convex lens; *E*, sample of epidermis; *T*, cover glass; *D*, diaphragm; *C*, collecting lens system; *P*, photocell.

[2] *This method suffers the objection that any fluoresced radiation, although of different wavelength from that of the incident beam, may be included in the estimated transmission for the given wavelength. However, all methods are susceptible to errors of this kind. The rather close agreement between our data for human epidermis and those of Lucas* (5), *obtained with a different method, suggests that this is not an important source of error.*

so that all the radiation passing through the aperture of the diaphragm is focused upon a vacuum photocell *P*.[3] The output of the photocell was fed into a linear amplifier (FP54 electrometer tube amplifier using the DuBridge-Brown compensated current). The output of the amplifier was measured by a recording galvanometer. The prisms and lens of the monochromator are motor-driven so that the radiation of various wavelengths illuminates the photocell successively. Thus, the continuous galvanometer record shows a peak corresponding to each line of the mercury arc, the magnitude of which measures the intensity of the radiation reaching the photocell.

In the first experiments the sample of epidermis was placed directly against the plane surface of the lens and covered with a thin cover glass *T*. The epidermis was placed in position, and the spectrum was made to travel across the photocell so as to obtain a complete galvanometer record. The epidermis was then removed, and a record obtained with the beam of monochromatic radiation striking the photocell directly. From a comparison of the two records, the transmission for each wavelength could be calculated. A possible error is introduced here, however, since changes of intensity of the source may take place during the relatively long period required for obtaining the two records, usually about 15 minutes.

This difficulty was avoided in later experiments by measuring the direct beam and the beam transmitted by the epidermis in rapid succession using only one wavelength at a time. In this case the epidermis was mounted on a disk of ultraviolet-transmitting glass and covered with a cover glass of the same material [4] approximately 0.1 mm. thick. The cell thus formed could be placed with the cover glass against the lens *L* so that the epidermis occupied virtually the same position as when mounted upon the surface of the lens. It was so mounted that it could be replaced instantly by a dummy cell optically equivalent to the first. With this arrangement, the measurement of the direct beam and the beam transmitted by the epidermis could be made for a given wavelength in rapid succession.

Due to the geometry of photocell and optical system, the greatest solid angle from which the radiation emerging from the epidermis can be collected is about 45°. In order to estimate the total transmission it is necessary to extrapolate to a solid angle of 180°, and to accomplish this, measurements were made of the radiation collected from three solid angles. Three diaphragms having apertures corresponding, respectively, to 14°, 27°, and 45° were arranged to be rapidly interchangeable at the position *D* (fig. 1). Measurements for the various wavelengths were made for each of these apertures as described above. In figure 2 the three curves labeled 14°, 27°, and 45° represent the spectral transmission of the mouse ear epidermis as measured for these three solid angles.

In order to check the validity of the extrapolation of such data for obtaining an estimate of the total transmitted radiation (180° solid angle), a second method was used. This employed a photronic cell with quartz window and a low resistance galvanometer as the measuring unit. It was possible to place the epidermis so close to the large plane surface of this cell, that the radiation could be collected from a solid angle of 150°. This method presents certain difficulties. The photronic

[3] *The sensitive surface of this photocell is cesium-cesium oxide on silver.*

[4] *All the optical parts of the apparatus were of quartz, with the exception of these disks and cover glasses which were of high silica glass, optically equivalent to quartz for our purposes.*

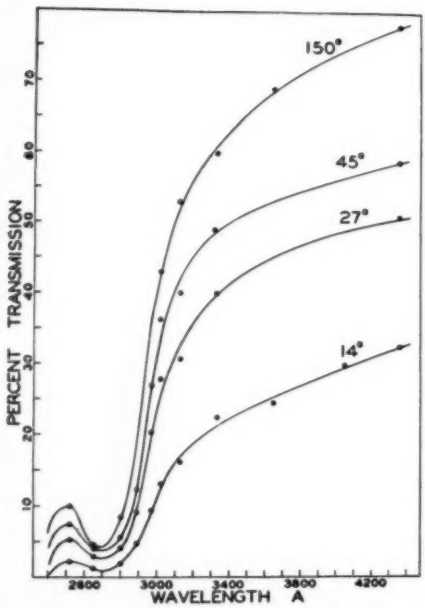

FIGURE 2.—Percent of incident radiation transmitted by epidermis of mouse ear, collected at different solid angles. The curves for 14°, 27°, and 45° were obtained by the first method, the curve for 150° by the second method. (See text.)

cell is so insensitive at shorter wavelengths that accurate measurements are difficult, and this greatly limits its usefulness. Moreover, its response deviates from the cosine law for radiation at high angles of incidence, which tends to give slightly low readings when used for the present purpose as does the fact that a solid angle of only 150° can be measured. The estimates obtained in this way are probably not far from the true values for total transmission, however, and represent the best obtainable with standard photocell equipment. They agree well with the values obtained by extrapolation from data obtained by means of the first method. If curves relating percent transmission to solid angles are plotted for any given wavelength there is no sharp break; i. e., a smooth curve drawn through the three points for 14°, 27°, and 45° obtained with the first method passes approximately through the point for 150° obtained with the second method.

In figure 2 measurements obtained with the two methods are compared. It is clear from inspection of this figure that increasing the solid angle from 45° to 150° increases to only a limited extent the amount of radiation measured. This is due to the increased path length at wide angles, which permits greater absorption. However, the ratio between the amount of radiation collected in a solid angle of 45° and a solid angle of 150° must vary with the wavelength because of differences in absorption by the epidermis. With very high absorption, most of the radiation scattered at wide angles will be absorbed before it leaves the epidermis, so that increasing the angle does not greatly increase the radiation measured. However, for the wavelengths important to this study a satisfactory value, which cannot be far from the total radiation passing through the epidermis, is obtained by multiplying by 1.3 the transmission value measured for 45° solid angle.

The mouse epidermis used in these experiments was obtained from the ears of strain A albino mice. The region of the ears is of particular interest for our studies since this is the site of most of the induced tumors (*1*). Moreover, the ears of these mice are relatively hairless, which obviates the necessity of depilation. The ears were soaked in 1 percent acetic acid from 24 to 36 hours, the solution being kept in the refrigerator during this period. At the end of this time the epidermis was stripped off from the underlying tissue, rinsed in water, and mounted for transmission measurements. The cover glass was usually sealed over the sample of epidermis in

order to prevent drying, which was found to decrease transmission slightly. Measurements on epidermis from different parts of the ears varied to only a limited extent.

The accuracy of the separation of epidermis from underlying tissues by this method has been adequately tested (*9*). The question whether epidermis so treated closely resembles living epidermis in its optical characteristics is one which needs some consideration, however. Bachem and Reed (*2*) found that the transmission of living rabbit skin was not greatly different from that of moist dead skin, and this in all probability applies in the present case. As far as absorption alone is concerned, little change is to be expected in the spectral region in which our interest centers, namely, wavelengths shorter than 3200A. Here, protein seems to be the principal absorbing substance (see p. 408), and although this is likely to undergo denaturation due to such treatment, no important change in chemical constitution is to be expected. The absorption spectra of proteins are little altered by denaturation, and such changes as might occur would probably not be detectable with our optical methods.

In order to rule out the possibility of alteration of the transmission characteristics owing to deformation of the cells, certain samples were soaked in isotonic saline instead of water, but no differences were detected. Since scattering probably occurs principally in the corneum this is not surprising since the scalelike elements of that layer would not be greatly deformed by soaking in water. The close adherence of the cells of the deeper layers due to protoplasmic bridges (*10*) would probably prevent marked changes in refraction at their boundaries, and hence changes in scattering. The true absorption should not be markedly altered by changes in volume of the cells.

For obtaining samples of human epidermis, resort was had to the vesicant action of solid carbon dioxide. A lump of that substance having a flat surface was applied to the desired area of the skin. A pale depression was observed immediately after removal of the carbon dioxide, but this was quickly replaced by erythema. Within 24 to 36 hours a blister had formed from which the epidermis could be cleanly removed with scissors leaving behind a smooth pale surface. Microscopic examination of sections and of whole mounts of the epidermis indicated a fairly clean separation with little or no trace of cells from the corium, as might be expected from the smooth remaining surface which showed no bleeding or thrombosed vessels. The basal cell layers appeared intact in most places, and had appreciable amounts been left behind, these would have been detected as areas of pigment on the surface from which the epidermis had been stripped. This method may be useful for other studies of epidermis. In the present instance it avoids the possibility of introducing extraneous light-absorbing material by the use of the more common vesicants.

RESULTS

In figure 3, a transmission curve for mouse epidermis is compared with a transmission curve for human epidermis obtained by Lucas (*5*). The curves are not strictly comparable, having been obtained under very different conditions. The human epidermis was cleared with acetic acid to reduce the scattering to a minimum, and the curve obtained more nearly reflects a true absorption spectrum than does that for uncleared mouse epidermis. Uncleared human epidermis does not transmit enough radiation of the shorter wavelengths to be measureable (see below), and hence cannot be used for the present comparison. It is obvious that the shapes

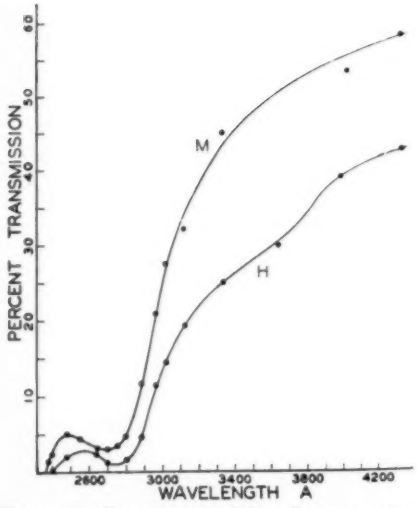

FIGURE 3.—Percent transmission of mouse epidermis collected at 45°, curve M; and of cleared human epidermis, curve H. (Curve H is from Lucas (*5*).)

of the two curves are quite similar. There is a minimum of transmission in each at approximately 2700A. Undoubtedly this is chiefly due in both cases to absorption by proteins of the corneum and the viable cells of the deeper layers, although the nucleic acids in the latter and possibly other compounds containing phenolic rings may contribute. A second maximum of absorption is represented by the complete abolition of transmission at about 2400A, and this is also characteristic of protein. There are minor differences in position of these absorption maxima in the two types of epidermis which probably have little significance; possibly they result from the difference in method of measurement. It seems safe to conclude that the absorption spectra of human and mouse epidermis are of the same general character in the region of 3000A, and that absorption in this region is principally due to protein in both cases. (See also Lucas (*5*) and Mitchell (*11*).)

Figure 3 gives a false impression of the relative transmissions of mouse and of human epidermis, since that of the latter had been greatly increased by clearing. Uncleared human epidermis, as used in our experiments, transmits wavelengths shorter than 3200A to a much less extent than does mouse epidermis. This is shown in figure 4, where the curves labeled H_1, H_2, and H_3 represent transmissions of samples of human epidermis, and curves M_1 and M_2 transmissions of epidermis of mouse ears. Curves H_2 and H_3 were obtained from two samples of epidermis taken from the same individual, whose complexion was intermediate between blond and brunet. Sample H_2 was taken from a volar surface of the forearm and showed a very mild degree of tan; while sample H_3 was taken from the dorsal surface of the forearm, which had been subjected to a moderately severe sunburn 2 weeks previous to the time the sample was taken and was deeply pigmented. Sample H_3 showed less transmission at all wavelengths than sample H_2 and did not transmit enough radiation of wavelength shorter than 3100A to be measurable. Sample H_1 was taken from the untanned volar surface of the forearm of another individual, likewise intermediate between the blond and brunet types. The transmission in this case is considerably greater than for the corresponding skin region of the first individual, but nevertheless shows little transmission below 3200A. Obviously the transmission of human epidermis varies considerably between individuals, and for different parts of the body. For comparison, a curve obtained by Lucas, using a comparable method, is shown by the dotted line in figure 4. The agreement is obviously good. Some of the curves of Bachem and Kunz (*8*) show greater penetration, as do those of Pearson and Gair (*6*) and Angus and Taylor (*7*). The

investigators last named, however, state that they dealt only with corneum. Throughout, there seems to be some uncertainty as to whether all or only part of the epidermis was included, the degree of pigmentation, etc.

Curve M_1 is for normal mouse epidermis and is similar to those already shown in figures 2 and 3. Actually the variation in transmission of epidermis obtained from normal strain A mice of comparable age was found to be relatively small. If such mice have been subjected to ultraviolet radiation, however, the transmission is greatly reduced, as is shown by curve M_2. The mouse that furnished this epidermis had been exposed to mercury arc radiation 5 days per week for 31 weeks, the daily dosage being 1.12×10^7 ergs per square centimeter of radiation shorter than 3200A (*12*), and a tumor had developed on one ear. The epidermis used for the measurements was taken from the contralateral ear, which showed only moderate, uniform thickening. Transmission of the epidermis from other irradiated mice was also found to be less than that of epidermis from normal mice.

It is worth remark that in the albino mouse, which does not develop melanin pigment, irradiation decreases the transmission of the epidermis. The change is undoubtedly due to thickening of the epidermis, particularly the corneum. Such thickening occurs in the case of man and is chiefly responsible for the reduction in sensitivity to sunburn which follows exposure to sunlight. It was at one time supposed that the simultaneously formed pigment accounted for this reduction in sensitivity, but its position in the basal cell layer of the epidermis is such that it would not provide effective protection against sunburn which results from more superficial changes. It may, however, be an important factor in preventing the penetration of ultraviolet radiation to the dermis of man. Since we are particularly concerned here with penetration of ultraviolet radiation to the dermis, as compared for man and mouse, it makes little difference whether melanin or the corneum is the principal absorber in the epidermis. Figure 4 shows that mouse epidermis, even from animals that have been repeatedly irradiated, transmits more radiation of wavelength shorter than 3200A than any of our samples of human epidermis.

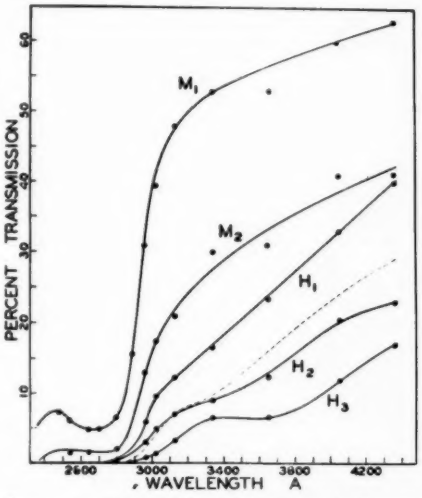

FIGURE 4.—Transmission of human and of mouse epidermis: M_1, from ear of normal young mouse; M_2, from ear of irradiated mouse; H_1, from untanned volar surface of forearm; H_2, from slightly tanned volar surface of forearm; H_3, from heavily tanned dorsal surface of forearm. H_2 and H_3 were from the same individual. All measurements for transmitted radiation collected at 45°. The dotted line is after Lucas (*5*) and is for a sample of uncleared human epidermis.

DISCUSSION

The fact that tumors of the skin of mice and rats may be induced by ultraviolet radiation has been widely accepted as conclusive evidence that sunlight is a major

causal factor in human cutaneous cancer (*13*). However, the majority of tumors induced in albino mice by ultraviolet radiation originate in the dermis (*1*), whereas virtually all tumors of human skin are epidermal in origin; and this might be thought to indicate that the two phenomena are not comparable. But if it can be shown that this discrepancy may be largely accounted for by differences in penetration of radiation in the two cases, the importance of such an objection is nullified.

In making comparison, it is not only necessary to consider the differences in transmission of human and mouse epidermis but also the nature of the radiation to which they are subjected. Sunlight, to which man is habitually exposed, has very different characteristics from those of the mercury arc, the source used to induce tumors in mice. In figure 5, the spectral distribution and intensity of sunlight within the tumor-inducing region, wavelengths shorter than 3200A, are compared with those of the mercury arc. The values for sunlight are such as may impinge upon human skin under conditions of maximum exposure, i. e., with a clear, dry air mass and the sun at zenith. Under average conditions not only would the total incident energy be less, but there would be a relative decrease of the shorter wavelengths. The intensities indicated for the mercury arc represent those incident under the conditions of most of our experiments, i. e., with the arc at approximately 50 cm. from the mice (*13*). The figure also indicates the portion of sunlight transmitted by tanned and by untanned human skin and the portion of mercury arc radiation transmitted by normal and by irradiated mouse skin.

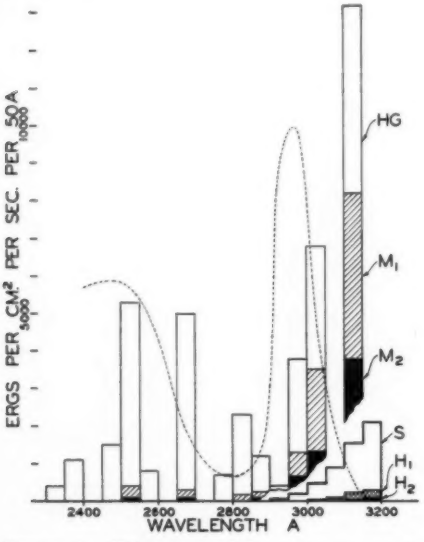

FIGURE 5.—*HG*, energy of the mercury arc; in most cases the 50A represented in the rectangle includes only one line. M_1 and M_2 represent, respectively, transmissions of normal and irradiated mouse epidermis. *S*, energy of sunlight with clear, dry air mass and sun at zenith (after Petit (*14*)). H_1 and H_2 represent, respectively, transmissions of untanned and tanned human epidermis. The transmission values are taken from figure 4, but have been multiplied by 1.3. (See p. 406.) The dotted line is the action spectrum for the erythema of sunburn.

The 3130A, 3020A, and 2967A lines of the mercury arc, each of which is represented in the diagram by a rectangle 50A wide, should be of particular importance in the induction of tumors of dermal origin in mice, for not only are they strong lines, but also they are transmitted to a considerable extent by mouse epidermis. For example, 40 percent of the 3020A line, which might for a number of reasons be expected to be the most effective of the three in inducing tumors, is transmitted by unirradiated mouse epidermis, while 17 percent is transmitted even by epidermis that has been subjected to many repeated irradiations. The amount of radiation that reaches the dermis is negligible for all the lines of shorter wavelength as com-

pared with these three most important lines. Rusch, Kline, and Baumann (*15*) found the 2537A line to be ineffective, which is readily explainable if it may be assumed that the tumors they produced were largely dermal in origin as in our own studies (*1*), for this wavelength penetrates only very slightly into the dermis. Experiments of our own now in progress also indicate that the 2537A line is relatively ineffective.

The total energy of sunlight is much less in this spectral region than that of the 3130A, 3020A, and 2967A lines alone of the mercury arc, at the distance used in our experiments. Moreover, the amount reaching the human dermis is very much less, only about 10 percent of the total being transmitted by untanned epidermis and only 1 or 2 percent by tanned epidermis. If cancer of the skin in man results from repeated exposure to sunlight, as is presumably the case, the latter may be taken as most representative of the type of skin affected. Moreover, if, as is to be expected, the effectiveness of the radiation falls off toward the long wavelength limit as it does in the case of sunburn (fig. 5), wavelengths longer than 3150A, which represent a large part of the radiation penetrating to the dermis, may have little tumor-inducing action.

When these various factors are taken into consideration, it seems unlikely that an appreciable amount of tumor-inducing radiation reaches the dermis of man under conditions which might be compatible with the induction of cutaneous tumors.

A much greater fraction of the incident radiation is absorbed in the epidermis of man than in the epidermis of the mouse. This in itself suggests that the incidence of epidermal tumors should be greater in the former. However, the relative amount absorbed by the living cells cannot be accurately estimated, since we are unable to differentiate absorption by the viable cells from attenuation by the corneum. Actually, there may be great differences in susceptibility of man and mouse, and of epidermal and dermal tissue, but the present studies alone give no information in this regard.

CONCLUSIONS

The amount of tumor-inducing radiation of sunlight which penetrates to the dermis of man is so small under ordinary conditions of exposure that, for this reason alone, production of tumors of dermal origin by this agent should be very rare. On the other hand, the absorption of virtually all such radiation in human epidermis makes it possible that this agent is an important factor in producing tumors of this site. This agrees with the fact that almost all human cutaneous tumors of exposed areas are epidermal in origin.

In the albino mouse, a large proportion of such radiation penetrates to the dermis, and it is probable that more is absorbed there than in the living layers of the epidermis. This agrees with the finding that the majority of tumors induced by ultraviolet radiation in these animals arise in the dermis, but are accompanied by a certain number which are epidermal in origin. The quantitative aspects have not been sufficiently analyzed to permit us to state whether or not this distribution can be explained on the basis of the relative amounts of tumor-inducing radiation absorbed in the different layers, or whether it is necessary to postulate important differences in the susceptibility of the different tissues to this agent.

The fact that tumors produced in albino mice by the action of ultraviolet radiation arise principally from layers which are not involved in human cutaneous cancer cannot be accepted as evidence that the two phenomena are not closely related.

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SUMMARY

A method for measuring the transmission of ultraviolet radiation through separated epidermis is described.

Wavelengths shorter than 3200A, which alone have carcinogenic action, pass through the epidermis of the albino mouse to a considerable extent. Transmission by the epidermis is less in mice which have been repeatedly irradiated than in normal mice, but remains relatively high. The production of tumors of dermal origin in these animals is accounted for by the large fraction of the incident radiation which penetrates to the dermis.

The transmission of these wavelengths by human epidermis is much lower than for mouse epidermis, and almost none reaches the dermis of well-tanned skin. This agrees with the fact that virtually all human cutaneous tumors of exposed areas are epidermal in origin.

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Methods for the Separation of Epidermis from Dermis and Some Physiologic and Chemical Properties of Isolated Epidermis[1]

By J. Percy Baumberger,[2] *research associate*, V. Suntzeff, *research associate*, and E. V. Cowdry, *director of research, Barnard Free Skin and Cancer Hospital*, and *professor of anatomy, Washington University, St. Louis*

A major project in the Barnard Hospital is to discover the changes that epidermal cells undergo when they become malignant after repeated treatment with methylcholanthrene. Thus far alterations in cellular and nuclear size (*1*), intranuclear viscosity (*2*), mitotic index (*3*), response to colchicine (*4*), and the influence on carcinogenesis of variations in the application of methylcholanthrene (*5*, *6*) have been investigated. In these studies the changes observed were localized in the epidermis microscopically by the study of sections.

The purpose of the experiments described in this paper was to improve methods for the separation of epidermis from dermis so that epidermis can be collected in a suitable condition for analysis by itself without the variable amounts of dermis which have in the past greatly complicated the interpretation of most chemical studies of the skin.

Others before us have realized the great handicap of being obliged to work with skin as a whole. Efforts to remove the epidermis mechanically have been only partly successful. Owing to the fact that dermal papillae often project up into the epidermis, it is almost impossible, even in the case of frozen skin, with a razor or a microtome knife to cut away the epidermis without taking some dermis. Berenblum, Chain, and Heatley (*7*) used a special planelike device to slice off the epidermis but found it necessary to correct their measurements of epidermal respiration by estimates made from stained sections of the amounts of dermis included.

In the leather industry, epidermis is removed from dermis by efficient chemical methods (Wilson (*8*)). These are, however, intended to leave a dermis suitable for leather production. The epidermis so separated would be useless for our work.

Separation of epidermis by maceration in dilute acetic acid was apparently first accomplished by Menschel (*9*). This method was later employed by Hoepke (*10*) to show the architecture of the proximal surface of the epidermis. Kooyman (*11*), working in our laboratory, made lipid analyses of epidermis isolated in this way. Cowdry (*12*) described the technique in detail, and it was later used to great advantage by Cooper and her associates (*13*, *14*, *15*) to provide whole mounts of epidermis in which mitoses could be counted much more easily than in sections. But treatment with 1 or even 0.5 percent acetic acid at a low temperature for 12 hours or more renders the epidermis useless for many kinds of chemical and physiologic analysis. Harris (*16*) made histamine analyses of epidermis

[1] *Aided by grants from the National Cancer Institute and the Jane Coffin Childs Memorial Fund.*

[2] *On leave from the Department of Physiology, Stanford University.*

removed by boiling water. For our purposes both the excessive temperature and the water are contraindicated. To study epidermis elevated by vesicants is unsatisfactory because the production of blisters involves chemical change and fluid shifts, and also because in many cases only the outer layers of the epidermis are raised by this procedure whereas all of it is desired.

MATERIALS

The skin employed was from the same strain of mice [3] (New Buffalo) used in our other studies on methylcholanthrene carcinogenesis. When it was desirable to obtain epidermis in large sheets, these were separated from human female breasts shortly after their removal in the operating room. For certain experiments white rats were used.

METHOD OF SEPARATION

Separation by Chemicals

The most probable explanation of separation by dilute acetic acid is that the acid brings about a swelling of the collagenic fibers which decreases their cohesive strength and, therefore, the binding of epidermis to dermis.

Collagen, as studied in "hide powder" (*8*), is very similar to gelatin in its behavior. It swells on either side of the isoelectric point so that at either pH 3 or pH 12 there is an increase in volume of about 100 percent. Consequently an alkali should be as effective as an acid for our purpose. We tried NH_4OH, because it has a relatively large concentration of undissociated NH_4OH and NH_3 molecules which penetrate quickly and change the pH to about 12. This rapid penetration is due to the absence of an electric charge on the undissociated molecules as shown by work reviewed by Jacobs (*17*).

It was found that after human skin had been soaked in N/1 NH_4OH for 20 minutes at room temperature the epidermis could be separated from the dermis completely and easily. When N/3 NH_4OH was used, 35 minutes was required before it could be as easily separated as after treatment with N/1 NH_4OH. Since N/3 NH_4OH is approximately isosmotic for mammalian tissue, it is to be expected that separation with it would be accompanied by a minimum change in volume of the epidermal cells.

In order to ascertain the minimum pH at which separation can occur, NH_4OH and NH_4Cl were combined in various proportions. The results are given in table 1. They show that relatively little increase in time is required as the pH diminishes and that even at pH 5.6, obtained with NH_4Cl alone, complete separation is easily effected after 24 hours' exposure. Even at relatively low pH values there is a considerable proportion of rapidly penetrating NH_3 and NH_4OH molecules which may change the local pH toward alkaline values and thus bring about a considerable swelling of collagenic fibers.

Separation by other chemical compounds was attempted (table 1), and Na_2CO_3 was found very effective although slower in action than NH_4OH. Boric acid buffers were very slow in action, and Na_2HPO_4 was unsatisfactory. N/1 HCl was also effective, but N/10 HCl had no effect even on long exposure.

A logical explanation of the effects of the compounds studied is that an acid or a base which can penetrate the tissue with sufficient speed and which will not in reaction with the tissue proteins form a compound having an acid dissociation

[3] *From New York State Institute for the Study of Malignant Diseases, Buffalo, through the kindness of Dr. W. S. Murray.*

TABLE 1.—*Separation of epidermis from human female breasts removed because of carcinoma*

BREAST OF COLORED WOMAN

Treatment	pH	Exposure	Separation
N/1 NH_4OH / N/1 NH_4Cl:			
10 parts:0 parts	11.3	10-12 minutes	Complete, except for some basal cells.
		20 minutes	Complete.
8 parts:2 parts	9.8	20 minutes	None.
		30 minutes	Complete, except for some pigmented cells.
7 parts:3 parts	9.6	20 and 30 minutes	Complete, except for some basal cells.
5 parts:5 parts	9.2	25 minutes	None.
		35 minutes	Partial, almost all basal cells remained.
3 parts:7 parts	8.9	40 minutes	None.
		1 hour, 5 minutes	Incomplete, some pigment on cutis.
2 parts:8 parts	8.6	1 hour	In small, localized sheets with parts of whole epidermis between.
		2 hours	Complete, except for basal cells.
0 parts:10 parts	5.6	6 hours	None.
		24 hours	Complete and very easy.
0.32 N NH_4OH isosmotic	11.0	20 minutes	Complete, but with some difficulty.
		35 minutes	Complete, easy.
Trimethylamine, 1 molar	11.0	40 minutes	Complete, with difficulty.
		1 hour	Complete, easy.
N/1 sodium cyanide	11.5	35 minutes	Complete, with difficulty.
		1 hour, 30 minutes	Complete, easy.
N/1 HCl	0	40 minutes	Complete.
N/10 HCl, N/1 NaCl	1	25 hours	None.

BREAST OF WHITE WOMAN

NH_4OH isosmotic	11.25	20-30 minutes	Complete.
N/1 NH_4OH / N/1 NH_4Cl, 7 parts: 3 parts.	9.67	37 minutes	Complete, except that everywhere 1 or 2 layers of cells remained attached.
		40 minutes	Complete.
		40 minutes and 1 hour	Complete, except that basal cells were left attached here and there.
		1 hour, 30 minutes	Complete, except that all basal cells remained attached.
Boric-KCl-NaOH-NaCl, isosmotic	7.95	4 hours	None.
		21 hours	Do.
		28 hours	Do.
	8.85	4 hours	Slight.
		21 hours	Fairly complete in pieces.
	9.60	3 hours	Partial.
		3.5 hours	Better, but not complete.
N/1 Na_2CO_3	10.3	50 minutes	Complete.
		40 minutes	Do.
M/10 Na_2CO_3	10.3	1 hour, 45 minutes	Complete, except for a few basal cells.
		2 hours, 45 minutes	Complete.
M/10 Na_2HPO_4	9.6	2 hours, 45 minutes	None.
		19 hours	Hardly any.

constant near the isoelectric point of collagen, will cause the collagen to swell and permit the detachment of the epidermis. Thus N/10 HCl is ineffective because H^+ions penetrate slowly, and at such a pH the number of H^+ions reaching the collagen would not be sufficient to shift the pH to 3. With N/1 HCl the tenfold increase in H^+would outweigh the slow penetration. The pH of slowly penetrating Na_2HPO_4 after reacting with tissue proteins would be too near the isoelectric point, whereas this would not be the case with Na_2CO_3 or NaCN.

In our experience the best method is to use isosmotic NH_4OH. The time required for the treatment of the excised skin of human beings is 35 minutes and of rats 20–25 minutes. Figure 1 illustrates how the human epidermis can be pushed off leaving behind a china-white dermis. It is interesting to note that the area of epidermis is always greater than that of the dermis from which it has been removed (table 2). The plane of loosening between epidermis and dermis is represented in figure 2. Projections of the epidermis fit accurately into concavities of the dermis and vice versa. Even when a projection is made up of a large body attached to the

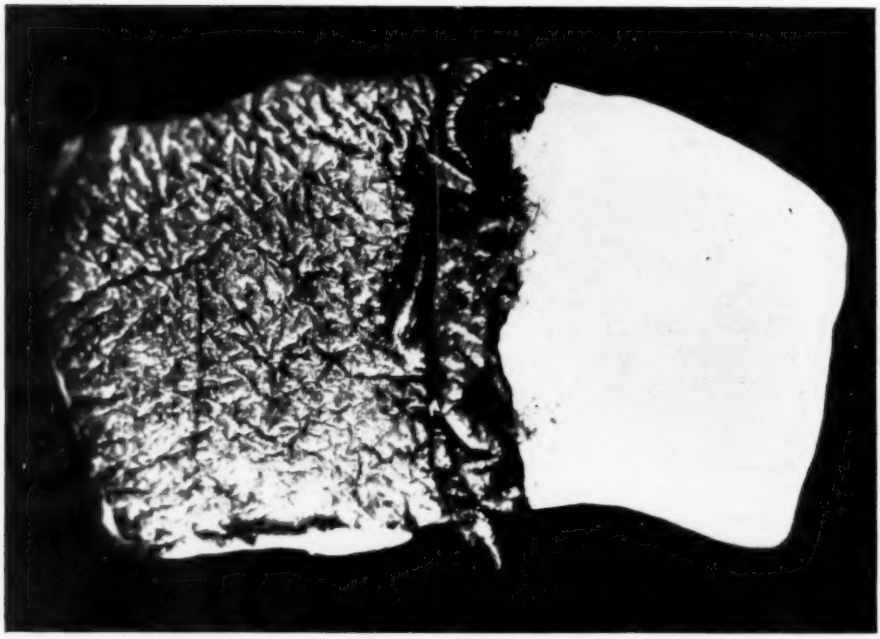

FIGURE 1.—Skin of breast of colored patient removed because of carcinoma. N/1 NH_4Cl solution, pH 5.6, 24 hours. Part of epithelium is rolled back and reveals the white surface of the dermis. × 5.

epidermis by a narrow neck, the body comes away from the encircling dermis with the epidermal sheet when the epidermis is detached. Either the dermis about the narrow neck stretches to permit the passage of the body of the epidermal projection or the girth of the latter is temporarily reduced. Probably both tissues yield to some extent.

By carefully gaging the time, the split

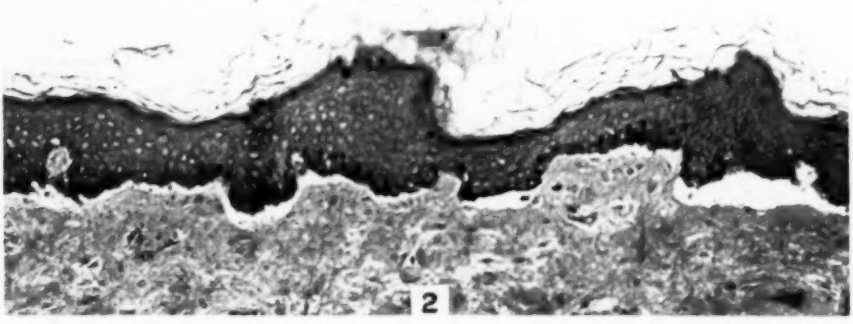

FIGURE 2.—Skin of breast of colored patient removed because of carcinoma. N/1 NH_4Cl solution, pH 5.6, 24 hours. Separation occurred between dermis and epidermis. It can easily be seen that pattern of epidermis exactly fits with pattern of dermis. × 165.

TABLE 2.—*Relative areas of dermis and epidermis removed after treatment with a chemical and with heat*

Material	Treatment with—	Exposure	Dermis		Epidermis	
			Square centimeters	*Percent*	*Square centimeters*	*Percent*
Human breast, colored female	N/1 NH_4Cl	27 hours	3	100	5.6	186
Do	N/1 NH_4Cl	do	5	100	9.5	190
Ventral aspects of forearm, white male	N/1 NH_4Cl	23 hours	3.8	100	6.24	164
Do	Heat, 50° C	2 minutes	3.42	100	5.0	146

can be made to take place immediately distal to the basal layer. The uniformity with which all except the basal layer can be removed is indicated by figure 3. The nuclei photographed stained unusually strongly with haematoxylin because in this specimen not all of the ammonium was washed out. The purpose of figure 4 is to show that even when the dermal surface is irregular, similar removal of all epidermis except the basal layer is nevertheless possible. Apparently after brief exposure to alkali, loosening is brought about in the proximal part of the spinous layer so that traction causes separation distal to the basal layer; whereas after longer exposure the loosening just proximal to the basal layer is greater, in consequence

FIGURE 3.—Skin of breast of white patient removed because of carcinoma. 7 parts NH_4OH+3 parts NH_4Cl (isosmotic solution) for 1½ hours. Distal part of epidermis separated very easily in one sheet leaving layer of basal cells attached to dermis. × 840.

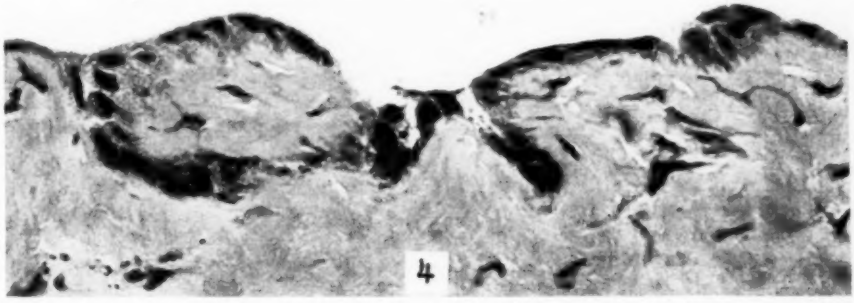

FIGURE 4.—Skin of breast of colored patient removed because of carcinoma. 5 parts of N/1 NH_4OH+5 parts of N/1 NH_4Cl for 1 hour. Basal cells are left attached to dermis even when the layer is very uneven. × 640.

of which traction causes separation not distal, but proximal to the basal layer and the whole epidermis comes off.

Extending our studies from freshly excised skin to the skin of living animals *in situ*, we found in this case, also, that separation of epidermis can easily be accomplished. The previously shaved skin over the abdomen of a rat was washed with soap and water followed by alcohol and ether. A gauze bandage moistened with 0.32/N NH_4OH was fastened to it. From time to time the ease with which the epidermis could be loosened was tested. After an interval of 35 minutes the epidermis of the treated area was removed in a complete sheet (under ether anesthesia), and the animal was killed. The time required exceeded by 75 percent that necessary for the removal of epidermis from excised rats' skins treated chemically in the same way. In all likelihood part of the NH_3 that penetrated the skin of the living animal was removed by the circulation, and this removal delayed the establishment of the necessary pH.

An improvement in the above-mentioned method of separation consists in protecting the skin, from which it is not desired to remove epidermis, with melted paraffin. The action of the ammonium can then be focused on a very small area.

We were able, for instance, to remove a papilloma from a living New Buffalo mouse, painted twice a week with 0.6 percent methylcholanthrene in benzene for 2¼ months, with a minimum of injury to the adjacent tissues. When later the papilloma and its dermal bed were examined in stained sections, it was seen that the line of separation between the two was very sharp. The isolated papilloma contained much less dermal tissue than would have been the case had it been cut off.

We then tested the viability of epidermis of a living rat, which had been loosened with NH_4OH and had therefore reached the stage when it could have been removed, but was left in position. The NH_4OH was washed away with 0.9 percent NaCl, and the condition of the epidermis was observed for 2 days. In a few minutes the erythema of the skin disappeared indicating that there had been no serious interference with its vascular bed in the dermis. The epidermis soon became affixed to the dermis so that it could no longer be removed. Perhaps this was brought about by a shrinkage of the collagenic fibers and a consequent regaining of their usual tensile strength. No indication of inflammation or degeneration could be seen grossly. It would therefore appear that at least the majority of the epidermal cells survived the treatment.

To obtain further data on the condition of epidermis which had been separated, its semipermeability to various substances was investigated. Epidermis removed by NH_4OH (or by heat as described in the next section) from human breast was spread over the opening of a glass tube into which was placed an indicator in water. The tube was then immersed in the solution to be studied. It was found that NH_3 and NH_4OH molecules, even from a mixture of NH_4OH and NH_4Cl having an acid pH, penetrated very rapidly into the interior through the membrane and changed the indicator (phenolphthalein) to pink in a few seconds. Na_2CO_3 also quickly made the interior alkaline, however, Na_2HPO_4 failed to penetrate. Table 1 (breast of white woman) shows that the penetrability is proportional to the length of time required to separate the epidermis from the dermis by these reagents.

Separation by Heat

Because an increase in temperature probably leads to a softening of the col-

lagenic fibers, temperatures much lower than the boiling point of water were used without the complicating factor of the water.

Specimens of whole skin from which the subcutaneous tissue had been removed were placed on a rectangular graded hot plate (such as is used in the mounting of paraffin sections) with the dermis down. The minimum temperature which would permit the mechanical removal of the epidermis in a short time was then ascertained. It was found that after exposure for 2 minutes at a position on the hot plate having a temperature of 50° C., the epidermis could be removed from human breast very readily and completely by blunt dissection.

Measurement of temperature by means of thermocouples [4] showed that the actual loosening of the epidermis from the dermis occurred exactly at 49.2° C. (126° F.). Incomplete separation was possible at 48.2°, but complete separation of the epidermis occurred at 49.2° with sudden ease and completeness. When the temperature is higher than 51°, more difficulty is encountered in the separation of the epidermis. This appears to be due to a general and more thorough softening of the collagen which permits separation even in the depth of the dermis.

The question naturally arises why the epidermis does not separate from the skin of a human being when exposed to temperatures considerably higher than 51° C. It is known that body extremities can withstand temperatures of 150°–200° in dry air with impunity for short periods of time. However, the temperature, even of the epidermis, probably does not approach that of the outside air in the living organism, for an efficient heat regulatory mechanism operates in the body and sweat is poured over the surface of the body. It is usually considered that water at 50°–51° will cause necrosis of the epidermis if applied for a sufficiently long time. That the temperatures of vesication should be as high as 71°–99° is probably due to the fact that this represents the temperature effective in a short surface application which, in order to produce vesication, must penetrate inward to the dermis and increase the permeability of the capillaries. Even if a temperature above 51° extended down through the basal layer of the epidermis, no loosening of the epidermis would be anticipated unless the permeability of capillary walls were increased; because, upon removal of the heat, the epidermis would again adhere to the dermis and would only be loosened by hydrostatic pressure developing from the capillaries. This reattachment of the epidermis to the dermis occurred in our experiment where, if we allowed the temperature to fall, the entire epidermis could no longer be removed with ease but left attached to the dermis practically all the basal layer, on the distal side of which the separation took place. This reattachment of the basal layer is to be anticipated if it merely depends on the resolidification of collagen, which is an entirely reversible process. Whatever took place to facilitate separation distal to the basal layer was not reversible, as separation of the outer part of the epidermis at this level was still possible after cooling.

Separation by this heat method is just as complete as by the ammonium and acetic acid techniques. Consequently it does not seem necessary to illustrate the results. The time and temperature required for mouse and rat skin are the same as for human skin. But when the heat-separated epidermis is studied in stained sections, there are definite signs, as might be expected, of loss of water. Though partial dehydration alone is not a serious

[4] *Dr. W. L. Simpson made the measurements.*

handicap for subsequent chemical analysis, it can be prevented by employing warm, moisture-laden air instead of the hot plate. The air is bubbled by pressure in a fine stream through water in a flask kept at a suitable temperature (80° C.) and escapes from an orifice a few centimeters away from the skin, which is pinned on a piece of cork. It impinges directly on the epidermis raising the temperature to 49.5°, when it can be peeled off.

COMPARISON OF OXYGEN CONSUMPTION OF EPIDERMIS REMOVED CHEMICALLY AND BY HEAT

The apparatus (fig. 5) used consisted of a glass tube (150×4 mm.) into which strips of the isolated epidermis were introduced in such a way as to have their entire inner surfaces exposed to air, clumping up of the tissue being avoided. It was felt that in view of the very small thickness of epidermis, adequate O_2 and CO_2 diffusion would take place. The tissue, if removed by NH_4OH, was first carefully freed from the reagent by washing with Ringer-Locke solution containing glucose until no increase in alkalinity appeared, and if removed by heat, was soaked in a similar solution. It was then freed of excess solution by touching it to a filter paper, but care was taken to have it moist while in the apparatus. At the other end of the tube containing the tissue a normal solution of $Ba(OH)_2$, held in place by asbestos fibers, served to absorb any CO_2 produced.

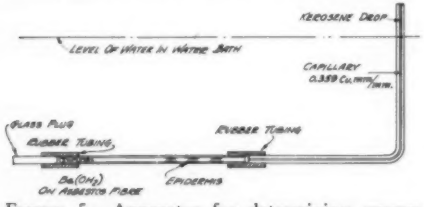

FIGURE 5.—Apparatus for determining oxygen consumption of epidermis.

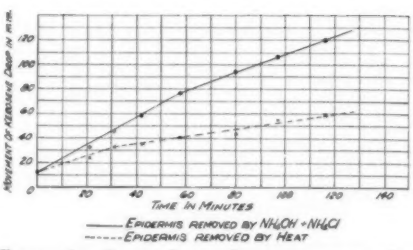

FIGURE 6.—Curve of rate of oxygen consumption of epidermis.

The tube was attached to a capillary at one end and was plugged at the other. It was next placed in a water bath at 37.5° ± 0.02° C. After sufficient time had been allowed for temperature adjustment to take place, a drop of kerosene was introduced into an upright arm of the capillary which protruded slightly above the water. As oxygen was consumed the kerosene drop moved far enough in the capillary tube to compensate for the change in volume. The excursion of the drop is plotted against time in figure 6. The bore of the capillary having been determined by mercury weighings, the volume of oxygen consumed per unit of time could be calculated. It can be seen that the rate of oxygen consumption remains constant over several hours and that the difference in rate between epidermis removed by two different methods remains constant. The oxygen consumption rate in terms of the total weight or the total nitrogen content of the tissue is given in table 3 for epidermis separated by different methods.[5] Our studies showed a high oxygen consumption rate for epidermis separated mechanically by a razor blade, without heat or NH_4OH, from mouse skin rendered hyperplastic by methylcholanthrene. When this rate is assigned the arbitrary value 100 percent, the rate for tissue separated by 7 parts NH_4OH : 3

[5] *The weighings and total nitrogen determinations were made by Lester Wicks.*

TABLE 3.—*Oxygen consumption of separated epidermis*

Source of epidermis	Treatment to effect separation	Weight of tissue	N		O_2 consumed per hour	O_2 per milligrams N per hour
		Grams	*Mg.*	*Pct.*	*Cubic millimeters*	*Cubic millimeters*
Hyperplastic mouse skin, 30–45 days	Chemical,[1] for 20 minutes	0.113	2.7	2.4	12.3	4.55
Do	Heat	.059	1.7	2.9	3.0	1.77
Do	Chemical [1] for 20 minutes	.068	1.9	2.8	7.77	4.1
Do	Heat	.150	2.72	1.82	5.75	2.11
Do	Chemical [1] for 30 minutes	.126	3.86	3.06	4.3	1.1
Do	Heat	.152	3.12	2.05	7.76	2.49
Do	Isosmotic NH_4OH	.229	3.62	1.58	4.67	1.29
Do	Heat	.104	2.86	2.75	5.4	1.9
Do	Mechanical	.025	.62	2.48	5.4	8.7
Human breast	Heat	.064	1.4	2.19	1.6	1.15
Do	Mechanical	.049	2.38	1.17	7.1	6.0
Do	Chemical [1] for 1 hour	.056	.93	1.67	2.16	2.33

[1] 7 parts N/1 NH_4OH to 3 parts N/1 NH_4Cl.

parts NH_4Cl is 37 percent; by heat, 23 percent; and by NH_4OH, 14 percent. Human epidermis shows this same order of diminution of respiration according to the means of separation employed.

Wohlgemuth and Klopstock (*18*) showed the respiration of razor-made slices of skin by the classical Warburg technique and found Q_{O_2}=cubic millimeters O_2 NTP consumed per milligram dry weight of pure epidermis per hour=−2.1. The deeper slices of skin gave Q_{O_2}=−0.4. The skin was obtained directly from operations for the foregoing experiments, but the skin from bodies dead 1 to 60 hours gave for epidermis Q_{O_2}=−2.0, e. g., showed very little postmortem damage to the respiratory enzymes. Since Q_{O_2} on the dry-weight basis would be expected to be about one-fourth of the respiratory rate calculated on a nitrogen basis, we may divide by 4 our value for mechanically separated epidermis and obtain rates which agree fairly well with those of the authors, namely, −1.5 for human epidermis, and −2.2 for hyperplastic epidermis of the mouse.

A very important point in connection with tissue like epidermis is to relate the respiration rate to some suitable reference base. Since the tissue very probably contains a considerable portion of keratinized cells, which presumably do not respire, a reference to dry weight, wet weight, or total nitrogen would in each case present objectionable features. Berenblum, Chain, and Heatley (*7*, *19*) used nucleic acid phosphorus as a reference base in their study of respiration of normal epidermis as contrasted with papilloma. This was a wise choice in view of the fact that keratinized cells ordinarily have lost their nuclei. It is interesting that when they used this reference, no difference in respiratory rate between normal cells and Shope papilloma cells was found, whereas when the dry weight was used a very great difference was observed. In conditions in which parakeratosis occurs the nuclei remain, so that even a nucleic acid phosphorus reference would be inaccurate. Work is under way in this laboratory to isolate keratinized material from the unkeratinized cells. The fact that pepsin does not attack keratin gives one means of separation. Another method is to take advantage of the difference in hydrolyzability of keratin as compared with other constituents of cells. In both the pepsin- and acid-digest methods of separating keratinized from unkeratinized cells,a leaching out of the chemical constituents of the former may occur, which would tend to vitiate the value of the chemical analyses. Perhaps a final

reference base cannot be found, and one must be driven to the use of several bases correlated with statistical analysis of the cellular constituents of the tissue.

DISCUSSION

The methods of separation described are not substitutes for the dilute acetic acid technique. The latter is distinctly superior for mitotic counts, for a study of the morphology of the epidermis, and probably for other purposes. By the acetic acid technique all the living epidermal cells are suddenly killed before the tissue is subjected to the slight mechanical manipulation necessary to remove it from the dermis. Consequently the appearance of the cells is more uniform than that of the cells in an epidermis separated by ammonium or by heat and subjected to various injuries unequally inflicted before they are fixed for sectioning and staining.

Through these new techniques it is possible that valuable data can be secured on alterations in permeability, oxygen consumption, and other fundamental properties that are modified during development and pathogenesis. In assessing the value of chemical analyses of specimens obtained in this manner consideration must be given to three principal factors: character of the sample, possibility of loss of material from the epidermis during separation, and possibility of chemical change within the epidermis during separation.

Character of the Sample

It is easy completely to remove the epidermal tissues by these methods up to the point in carcinogenesis in our series, when clumps of epidermal cells become malignant, invade the dermis, and thus become detached from the epidermal sheet. These clumps remain in the dermis and cannot, therefore, be included in the analysis. The tissue separated consists of epidermis in the strict sense, and of hair follicles and sebaceous glands, if the latter two components are present. Sweat glands do not introduce a complicating factor in mice because they are absent. Histologic examination of tissue in close apposition to that analyzed and possessed of the same gross characteristics is essential to the interpretation of the results of analysis because the frequency, or volume, of hair follicles and sebaceous glands as well as the degree and extent of keratinization must be taken into account.

Possibility of Loss of Material From the Epidermis During Separation

Since the skin is quickly excised, removal via the blood stream is negligible. The NH_4OH solution is isosmotic, yet it will disturb the electrolyte equilibrium of the cells somewhat and it cannot be estimated how near to normality this equilibrium can be restored by the process of washing out the NH_4OH with Ringer-Locke glucose solution. Consequently the heat method of separation is preferred. This is now being used by Carruthers and Wicks preliminary to determinations of nitrogen, iron, and other substances. Though dehydration of the epidermis can be prevented by applying heated vapor to the surface, this is not necessary for the reason that loss of water is not a great disadvantage if the reference base is neither weight nor volume.

Possibility of Chemical Change Within the Epidermis During Separation

It is likely that heat causes some denaturation of protein since the temperature used is above the heat of coagulation of some cell proteins. That this denaturation does not go very far is proved by the continuance of respiration which shows that respiratory enzymes are still in part functioning. Ammonia is too weak an alkali to denature protein, and this type of modification of the cell should

therefore be small in tissues separated by our chemical method.

SUMMARY

Epidermis can be loosened from the underlying dermis by treatment of the skin with isosmotic NH_4OH for 35 minutes or by increase of temperature to 50° C. for 2 minutes. It can then be easily removed in a complete sheet by blunt dissection. In the case of human skin, only the lower portion of the hair follicles and the deeply seated bodies of the sweat and sebaceous glands remain in the dermis. In epidermal methylcholanthrene carcinogenesis of mice, these methods yield complete sheets of epidermis plus appendages up to the time when the epidermal cells break loose and invade the epidermis.

The epidermal tissues thus treated are not killed, but are only temporarily loosened by both NH_4OH and heat. If not promptly removed, they become again affixed to the dermis. Epidermis, isolated by both the NH_4OH and heat methods and placed in respiration chambers, continues to consume oxygen. For some kinds of chemical analysis, the heat technique is superior becasue the tissue is not brought into contact with any aqueous solution and there is no loss of material from it. But by the NH_4OH technique either the entire epidermis or the epidermis distal to the basal layer of cells can be removed. An opportunity is thus afforded for a chemical comparison of the basal layer with the whole epidermis.

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Effect of Certain Sulfur-Containing Compounds on the Initiation of Mitosis in Amoeba proteus

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INTRODUCTION

An outstanding characteristic of tumor growth is the continuing proliferation of cells under conditions in which normal tissue cells show very low or no such activity. It is, therefore, evident that the study of any agent or agents capable of accelerating cellular proliferation, especially if the agent or agents are of general occurrence in organisms, is of direct interest in cancer research.

There is extant a considerable mass of evidence, including the well-known researches of Hammett and his coworkers, several papers by Voegtlin and the writer, and papers by Jahn (*1*), Reimann (*2*), and others, which shows that exposure of cells to relatively low concentrations of a number of sulfur-containing compounds results in accelerated cell proliferation. Certain sulfur-containing amino acids and in particular the tripeptide, glutathione, have been shown to be effective in concentrations as low as 10^{-3} or even 10^{-8} mols per liter.

In all the experimental data that have come to the writer's attention the period of exposure is, relative to the life of a single cell, a long one, so that all or at least the major part of the life cycle of the cells studied, and in many cases several cell generations, pass during the experimental period. This brings up the question whether the action of these stimulants to cell division is concerned specifically with the fission processes or merely acts as a general growth stimulant. The work done in this Institute to date reveals no stimulating action of such substances on the phases of mitosis (Chalkley and Voegtlin (*3*), and unpublished work by the writer). There is, however, evidence that growth processes leading to increase in fission rate are affected. Chalkley and Voegtlin (*4*) pointed out that the effect of —SH glutathione was a function of the cell volume at the time of application of the reagent, and also that the percentage rate of increase in nuclear volume was greater in M/100,000 —SH glutathione than in control saline. Chalkley and Voegtlin (*3*) showed further that under a variety of conditions the rate of increase in nuclear volume in the cell is directly correlated with the increase in fission under survival conditions. (In Chalkley and Voegtlin's experiments, *Amoeba proteus* was used.) It is again suggested, as has been done previously (*3*), that the facts indicate that one role played by the sulfur compounds, particularly those containing the labile —SH or —SS groups, is to act as, or to help form in the cells, activators for enzymes especially those concerned with nuclear growth, and thus enhance that growth and consequently stimulate fission.

Can these labile sulfur groups act directly upon the cell mechanism to initiate fission or do they act indirectly assisting the cell to form metabolites requisite for the process? To reach any decision on this point, it would be necessary to perform an experiment under such conditions that a change in the rate of the entry into fission

in a given group of cells could be made clearly evident and measurable in a very short time. The decision as to whether a reagent probably acts directly or indirectly on a process occurring in a living cell must of necessity lie in the length of time elapsing between exposure to the reagent and the appearance of the response and, as will be seen, in the nature of the response.

A method was devised which permitted detection and measurement of very rapid changes in the rate of entry into mitosis in *Amoeba proteus*. The details of the method, together with the results obtained with respect to the effect of certain organic sulfur compounds, are here reported.

MATERIALS AND METHOD

Amoeba proteus has an interkinetic period averaging 48 hours. If a group of adult cells be selected from a culture, the rate of fission in that group will be very high indeed. Under the conditions used, in a group of 500 adult cells the observed rate [1] was from 0.6 to 1.0 cells per minute. The selection of such a group of cells, however, is a matter of considerable difficulty. The most important factor is the provision of vigorous cultures of great uniformity. The method of obtaining such cultures is given in detail since the maintenance of the experimental conditions has been found to be essential.

The amoebae were from a clone culture maintained in this laboratory since June 1928. During this long period of culture and by reason of frequent transfers, the cultures now contain bacteria, mold, one ciliate (*Colpidium* sp.), amoebae, and no other organisms.

The cultures are maintained in glass moist chambers about 17 cm. in diameter and are set up as follows: The moist chambers are washed with 50 percent nitric acid, rinsed with tap water 15 times, and finally rinsed with distilled water 2 or 3 times, and dried. A layer of neutral agar-agar is then placed on the bottom of each dish. A solution of 3 percent agar-agar is first prepared and filtered through cotton, neutralized to pH 7.0, and autoclaved. This is tubed as convenient, about 15 cc. per tube is usual. Two tubes (30 cc.) are melted in a water bath and when completely fluid are poured into the bottom of the moist chamber, which must be well-warmed, and are evenly spread by manipulation. Then, before the agar has set, 6 grains of polished rice (a soft grain rice; the very hard grain rices are unsatisfactory) are pressed separately into the soft agar-agar about 1 or 1½ inches in from the periphery of the dish, and approximately equidistant from each other. The chamber is then covered, placed on a level surface, and the agar allowed to set for at least 2 hours. (A shorter period results in the loosening of the agar plate later on.) After the 2-hour wait, 200 cc. of saline solution containing 0.1 gm. NaCl, 0.004 gm. KCl, 0.006 gm. $CaCl_2$, and 0.004 gm. $NaHCO_3$ per 1,000-cc. glass distilled water is poured gently into the chamber, and it is ready for seeding with amoebae.

For seeding, a large number of amoebae are removed from a vigorous culture with a fine pipette and put into a small flat-bottomed glass dish containing 15 to 20 cc. of the saline solution, which hereafter will be referred to simply as saline, and allowed to settle. The amoebae will adhere strongly to the glass bottom within 20 to 30 minutes. When the amoebae are well-attached, the saline is decanted rapidly, the dish refilled with saline, and the amoebae are again allowed to settle. This process should be repeated until the amoebae when inspected under a low magnification (approximately 30 diameters) are seen to be free of food organisms and detritus. The amoebae are then lifted by means of

[1] *The rate uncorrected for the number of cells used.*

a fine pipette and carefully placed, 50 to each rice grain, in the new culture, and food is added. The food is prepared as follows: With a fine pipette a few cubic centimeters of the culture fluid, taken from a point in an old culture where a large number of food organisms are congregated, is filtered into a small dish through a little cotton to remove mold strands and coarse detritus. The contents of this dish are diluted until 0.01 cc. is found to contain from 2 to 10 food organisms. Then sufficient of the diluted fluid to contain an average of 10 organisms is placed in the center of the new culture.

The culture should be disturbed as little as possible until ready for use (about 9 days) and should be kept at a temperature of from 24° to 26° C. In the experiments here reported all work was done and the cultures were maintained in a constant temperature room held at $25° \pm 1°$. A variation of 5° in temperature, particularly a rise, will so interfere with the development of the cultures as to impede seriously experimentation. In the one or two instances in which it occurred during these experiments it necessitated stoppage of experimentation until new cultures had matured.

A culture thus prepared can be used from the eighth to the thirteenth or fourteenth day after seeding. The standard practice during the experiments was to select cells from cultures from about the ninth to the twelfth day. New cultures were seeded on Tuesday and Friday of each week.

Selection of the Cells

The selection of cells from these cultures is to a large extent a matter of experience, and any one who attempts use of this method must expect to undergo a period of self-training before satisfactory selection can be made. However, certain criteria can be laid down which will assist. Inspection of the cultures will show that the rice grains are covered with veils of mold at the end of the eighth or ninth day and are surrounded by a dense cloud of actively swimming food organisms which occupy dome-shaped spaces 2 to 3 cm. in diameter centered about the grains. Surrounding each space will be found a ring of amoebae.

If this ring of amoebae is inspected under a magnification of about 40 diameters by means of a binocular dissecting microscope and by using reflected light, the conformation of the cell surface, as well as the general form of the cells, can be discerned. Eighty to ninety percent of the cells approaching fission are quiescent, somewhat rounded in general outline, and with the entire surface papillated with rather fine and uniform pseudopodia. Not all cells exhibiting these characters, but very few that do not, will divide soon. Furthermore, of the cells having such a form those chosen from an area in the culture where a number of fissions are taking place [2] are more likely to divide soon. Since volume is a factor of considerable, though not critical importance, cells that approximate in volume the dividing cells in the same culture will serve best. No cell not attached to the substrate should be selected. Figure 1 shows the types of cells to select and, more important, the types to avoid.

Care was taken to secure pure reagents. The copper-free glutathione and the cysteine were prepared in this Institute by Dr. J. M. Johnson. The cystine disulfoxide was furnished through the kindness of Dr. Frederick Hammett, of the Lankenau Hospital, Philadelphia, and contained but 0.5 percent cystine. The β, β'-dithiodipropionic acid and cysteic acid were specially prepared by Dr. J.

[2] *For the criteria of fission see Chalkley and Daniel* (5)

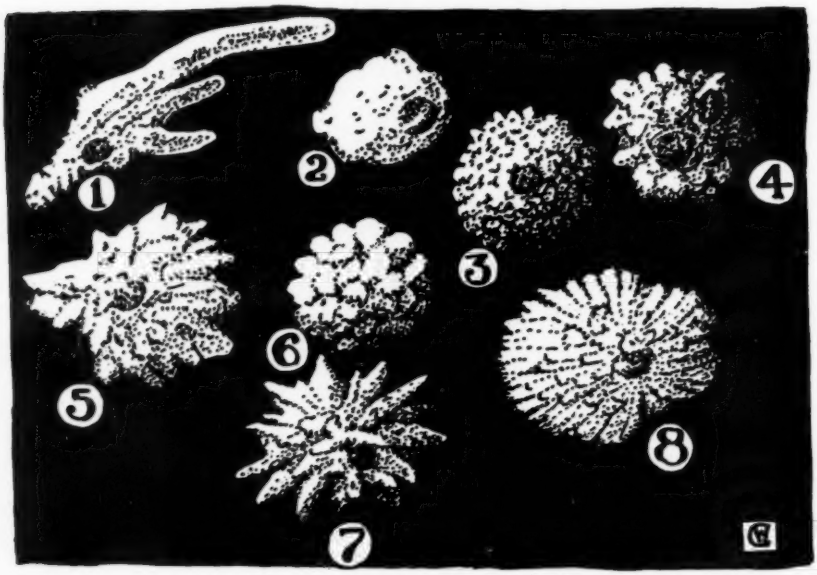

FIGURE 1.—Forms of *Amoeba* to be accepted or rejected in making selection. *1*, Normal locomotion; *2*, Moribund form, note smooth outline and lack of pseudopodia; *3*, In fission, prophase; *4*, With large food vacuole, quiescent state correlated with food intake; *5*, Quiescent form, slightly stellate; *6*, In fission, anaphase; *7*, Stellate form, quiescent; *8*, Rounded, flattened form with many fine pseudopodia, particularly likely to enter mitosis in a short time. Reject *1* to *4*, and *6*, accept *5*, *7*, and *8*.

White, and the diglycylcystine and cystinyldiglycine by Dr. J. P. Greenstein, both of this Institute. The cystine used was of first grade secured from the Eastman Laboratories.

EXPERIMENTAL PROCEDURE

Each experiment consisted of a series of 32 tests. In each test 20 cells were selected and lifted from the culture with a fine pipette, and placed in a small glass dish in about 5 cc. of saline solution; from these 16 were selected and placed 8 to a dish in 2 glass dishes each containing from 1 to 3 cc. of saline, the amount depending on the experimental solution to be used. After this they were kept under observation for 3 to 4 hours. At a predetermined time during the 4-hour period, constant for each experiment, a sufficient amount of the reagent used in the particular experiment, which had previously been dissolved in saline, was added to each dish to bring the molar concentration of the total fluid content to a desired value. The time at which any cell entered into mitosis was noted and recorded for each test period of 4 hours. After all tests had been completed, these times were plotted as a single cumulative curve for one 4-hour period. The slope of this curve gives, of course, the observed rate of initiation of fission in entries into mitosis per unit time during the experiment.

To obtain a true value for the rate, the number of cells used must, of course, be taken into account. The curves obtained can, for the purposes of analysis, be presented as straight lines.

The use of he straight line in the

analysis of the curves is based on the following considerations: It is very obvious that some of the experimental data might well be represented by other types of curves; in all, regions exist which very closely approximate straight lines and which are connected by regions where the curvature changes with great rapidity. It is likewise obvious that the rapid changes that occur at these regions are not instantaneous; but no great error is involved in representing them as discontinuities in the rate curves rather than sharply flexed sigmoids, as they would appear if more accurately represented. As will appear in some cases, noticeably in the curves obtained with 66×10^{-6} mols (M/15,000) and 200×10^{-6} mols (M/5,000) GSH, the question may well be raised whether on the data of the individual experiments the representation should not be made by a single line rather than two or more. The writer believes, however, that the decision must be made with due consideration for the breaks in the curves that appear definitely in the intervening concentrations. In any case if such decision should later prove erroneous, it will not affect the main conclusions drawn.

It is therefore sufficient to take $\frac{\frac{\Delta x}{\Delta t}}{N}$, where $\frac{\Delta x}{\Delta t}$ represents the calculated slope, and N the number of cells still unchanged at any point on the line, to obtain the actual rate at that point. If these values are plotted against time, a line drawn through the plotted points will show the change of rate with time and the rate at any time will be the ordinate value at that time.

To avoid confusion between the observed rate and the actual rate in the discussion, the former is hereafter referred to as the slope of the experimental curve, or simply the slope, and the latter is designated the rate of entry into fission, or simply the rate. In all curves the slope was calculated from the observations, after averaging by fives (this seems permissible since on the ordinate the observations are continuous) by using the method of averages given by Lipka (*6*). The rate curves were calculated by taking $\frac{\frac{\Delta x}{\Delta t}}{N}$ at convenient points on the calculated curves plotting the values obtained against time, and passing lines through the points thus determined. The full data are presented in all instances so that the reader may form his own opinion as to the validity of the procedure.

Control Curves

The rate of entry into fission of a given group of amoebae during a short period of time may be assumed to depend on several factors: temperature, cell volume, pH, condition of culture, and physiologic age of the cell. If these factors are controlled, it might be expected that the rate of entry will for a given group be constant or very nearly so. The initial experiments were therefore designed to determine if this is so. Experiments were performed using amoebae in saline and making no change in the saline during the course of the experiment. Each curve in this, as in all experiments except when noted, is based on the observation of 512 amoebae.

The results are presented in figure 2. It will be noted that there is an initial reverse flexure in the curves during the first 80 minutes or so. All curves then straighten out and are approximately straight lines of various slopes for the remainder of the experimental periods. It appeared likely that the initial changes in rate of fission were due to transfer from the culture to the saline used during the observations. An experiment was there-

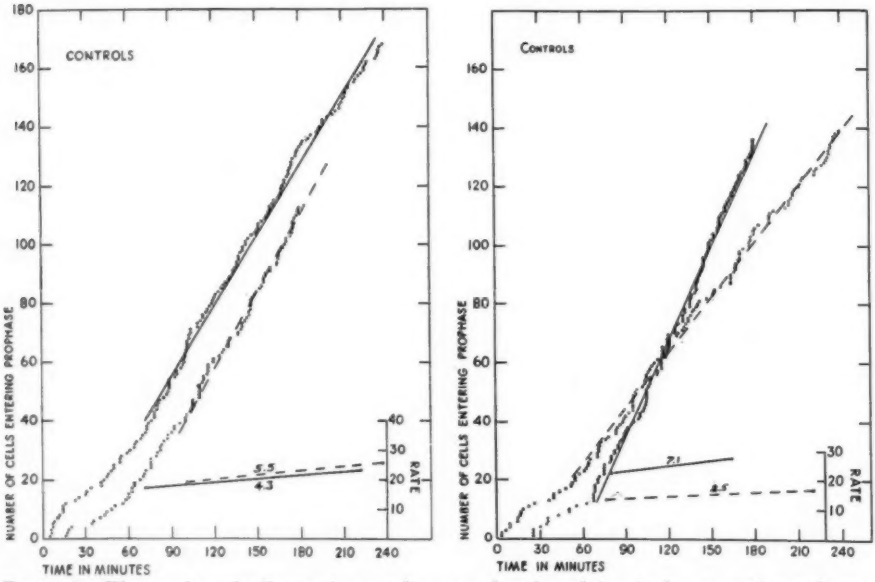

FIGURE 2.—The number of cells entering prophase as a function of time in four control experiments. Rate unit is change per cell per minute$\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

fore performed in which the observations were carried on in the culture. The dishes used were immersed in the culture close to the rice grains and allowed to stay for 24 hours to reach equilibrial conditions as nearly as possible. The amoebae selected were merely lifted within the culture and placed in the dishes for observation. The result is shown in figure 3. The initial flexure is less, which tended to confirm the assumption. The remaining effect is presumably the result of handling the cells. That mechanical stimulation in early fission will result in delay is a common experience as noted by Chalkley and Daniel (*5*) in previous work. Apparently the effect is noticeable before the observable phenomena of fission have made their appearance. After this preliminary period of adjustment, all these control curves are practically straight lines.

It is at once obvious that the rates are all accelerated and not constant. The rate for each curve was calculated from a straight line fitted to the points as above described. The rates are given here and in all experiments as calculated in terms of change per cell per minute$\times 10^4$.[3] The accelerations are given per cell per minute per 100 minutes$\times 10^4$.

In the curves two different types of effect obtained: (1) Changes in rate that in the plots appear as discontinuities, possibly more properly represented by steeply sloped sigmoid curves (these are regions where the rate varies rapidly and with varying acceleration); and (2) changes in rate that vary much more slowly, and, within experimental determination, with constant acceleration. The first type of change will be designated a stimulation or depression phase depending on the direc-

[3] *This, of course, may be read as cells per minute per 10,000 cells.*

tion of change, and the second an acceleration phase.

It will appear that in all experiments the adjustment period extends from 60 to 90 minutes, and that in all the cells settle down to an acceleration phase. The acceleration varied from 1.2 to 10.0 and averaged 4.2. These experiments throw no light on the reason for this initial acceleration. It must be ascribed, however, to conditions existing in the culture, since in the experiment performed in the culture this acceleration was 7.0. As pointed out, the cultures used are particularly vigorous, i. e., growing rapidly so that a steady acceleration of division as here indicated may obtain by reason of a steadily increasing food supply or other beneficial conditions. The adjustment period is, then, due in part to mechanical shock in handling and in part to a rapid though slight change in environment on transfer from the culture medium to the saline solution.

Consideration makes it obvious that, after setting up a given group of cells, allowing a period sufficient to cover that required for the initial adjustment and for the establishment of a phase of uniform acceleration of initiation of fission, if a single variable (i. e., a reagent) capable of changing that rate is introduced and allowed to act during the remainder of the experiment, a plot of the results should show a change of slope occurring either subsequently to, or coincidentally with the moment of entry of the variable. The time relation will depend upon the rapidity of the action of the variable on the system, cell plus original environment.

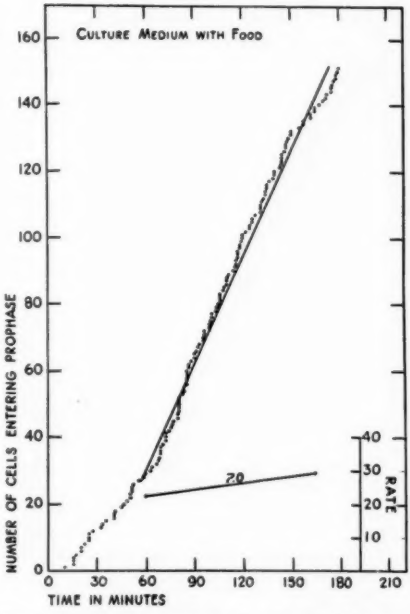

FIGURE 3.—The number of cells entering prophase as a function of time in an experiment performed in a culture. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

EFFECT OF CYSTINE

An experiment was therefore set up to test the action of cystine. All glass dishes used contained 2 cc. each of saline, and the amoebae, together with a minimum amount of saline, were transferred to each of them by use of a capillary pipette, thus ensuring only a negligible change in the volume of the saline in each dish. After 90 minutes, 2 cc. of an M/5,000 solution of cystine in saline was added to each dish bringing the cystine concentration in the dishes to M/10,000. Records of entry into fission were kept throughout the experimental period of 3 hours. Thirty-two tests using two lots each of eight amoebae were made, and the summed results were plotted (fig. 4). It will be seen that about 30 minutes after the addition of the cystine the curve flexes sharply upward, indicating stimulation after a latent period of approximately 30 minutes. A duplicate experiment was performed to check this result with similar results (fig. 4). The point of

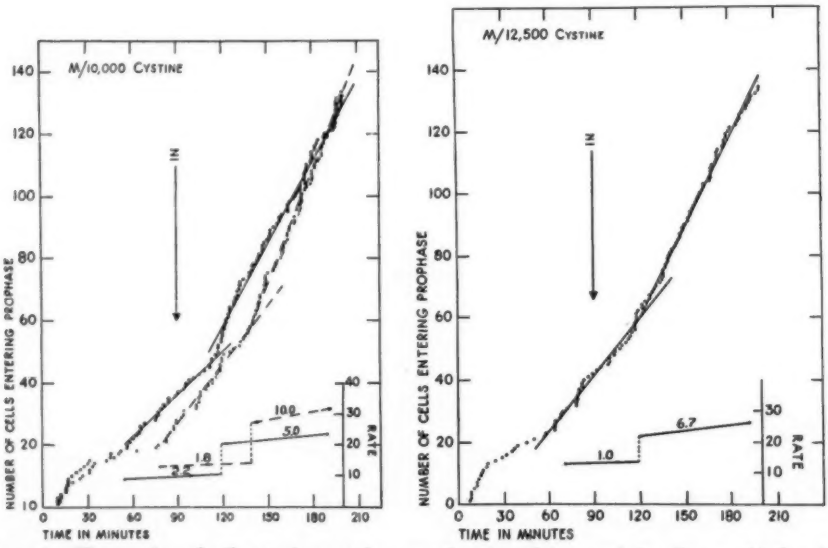

FIGURE 4.—The number of cells entering prophase as a function of time, and the effect on this function of addition of cystine to the environmental fluid. Corresponding curves for entry and rate are shown with identical symbols. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

deviation of the curve, however, is somewhat obscure but falls evidently between 28 and 40 minutes after addition of the cystine. The results for a similar experiment in which cystine in a concentration of M/12,500 was used is also shown in figure 4.

The data in each experiment can be well described by two straight lines intersecting approximately at the points of flexion noted. On passing these through the points and calculating from them the rate curves, values for rates and accelerations were obtained (fig. 4). This shows that on exposure to M/10,000 cystine, after latent periods of 28 and 48 minutes, respectively, the rates were raised very suddenly (stimulation) 9.8 and 13.1 units, and the accelerations following were increased above those prior to the stimulation by 2.8 and 8.2 units, respectively, in the two experiments. The lower concentration, M/12,500, had a similar effect, the latent period being 29 minutes, and the changes in rate and acceleration 8.3 and 5.7, respectively. A glance at the M/10,000 curves will show that because of local variation in the experimental curves the points of flexion cannot be accurately posited in time. The data evidently do not permit any statement as to the effect of concentration in these experiments. The amounts of stimulation and increase in acceleration are, however, significant.

EFFECT OF CYSTEINE

The effect of the reduced form of the amino acid was next ascertained. In the first experiment, cysteine was used in M/12,500 concentration. In curve I, fig. 5, *A*, there is a sharp downward flexion 10 minutes after the introduction of cysteine, showing a depressing effect. The

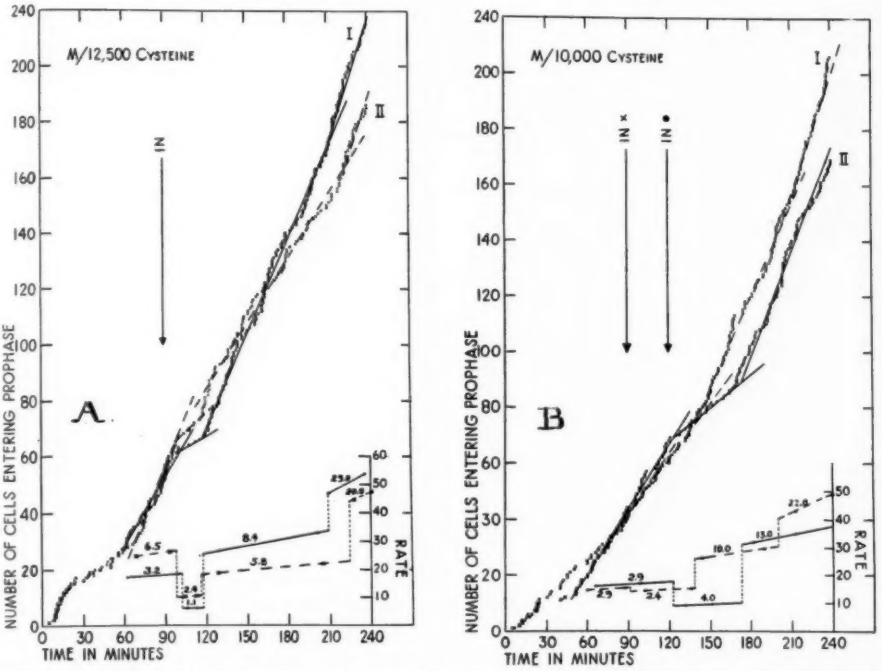

FIGURE 5.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of cysteine to the environmental fluid. Corresponding curves for rate and entry are shown with identical symbols. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

duration of this effect is, however, very short, and recovery (indicated by an upward flexion) occurs in 20 minutes, after which the slope is constant for about 100 minutes, then there is a second upward flexion to a new slope which holds to the end of the experimental period. The shortness of the depression was such that it might well be due to experimental error so a second trial was made (curve II, fig. 5, *A*), which confirmed the first. The next experiment was performed using cysteine in M/10,000 concentration and the period was extended to 4 hours. Curve I, fig. 5, *B*, shows that with the increase in concentration, the depression appears almost instantaneously on addition of the reagent, and that recovery does not begin for a period of 50 minutes. Again recovery is shown by a quick upward flexion to a higher slope than existed prior to addition of the cysteine and, as before, is followed after an interval by a further flexion to a still steeper slope, i. e., depression is followed by stimulation. A second experiment was performed in which the cysteine was added later in the experiment (curve II, fig. 5, *B*). Recovery is indicated, but the experiment was terminated before the stimulation period was reached. This curve, however, shows the initial depressive effect very clearly. A statistical test shows the changes to be very significant.

These changes become very apparent in the rate curves. From these it is seen that in the first two experiments on exposure to

M/12,500 cysteine, after latent periods of 8 and 12 minutes, respectively, the rates drop suddenly by 12.4 and 16.8 units, and the accelerations drop from 3.2 and 6.5 to 1.1 and 2.4 units; then after depression periods lasting 18 and 16 minutes, a sharp recovery of rates of 19.5 and 7.6 occurs, and the accelerations increase to 8.4 and 5.8 units. This recovery lasts for 109 and 92 minutes and is followed by a further stimulation of 13.2 and 20.8, the accelerations finally rising to 25 and 20. This complicated series of changes is also apparent in the respective rate curves for the two M/10,000 cysteine experiments. Here the latent period is, however, reduced to zero, the drops in rate are 8.6 and 1.5, the depressions last longer, 50 and 48 minutes, with accelerations of 4.0 and 2.3. The rises in rate at recovery are 21.1 and 10.7, and the accelerations change to 13.0 and 10.0. In the one curve where the final stimulation phase was reached, the rate rose by 10.0 and the acceleration went to 22.0. The possible significance of this complex picture will be discussed later.

Effect of Methionine

Three experiments were performed using methionine as the reagent in M/10,000 concentration. All were completely negative during the period used, 3 hours. The rates and accelerations were well within the range of variation of those of the controls, and no reproducible change in either rate or acceleration could be demonstrated, as will be seen in figure 6.

Effect of Reduced Glutathione

Since so much importance has been attached to glutathione as a well-known and much-investigated component of tissues, it seemed well to study its action more closely, particularly as it seems quite probable that this tripeptide may occur and function as a chemical individual in organisms, while

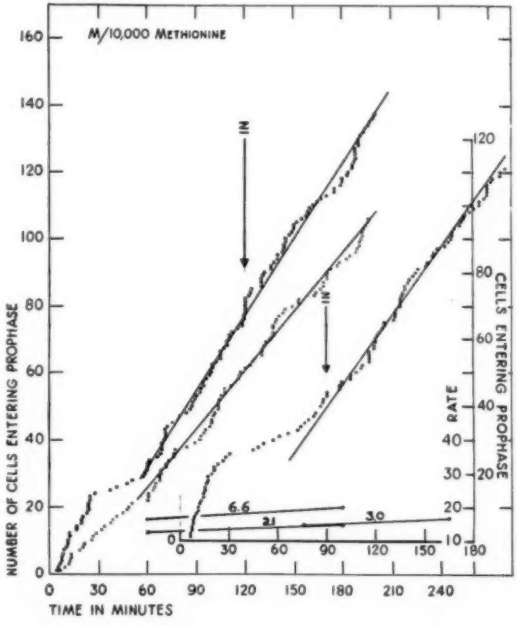

FIGURE 6.—The number of cells entering prophase as a function of time, and the effect on this function of addition of methionine to the environmental fluid. Corresponding curves for entry and rate are shown by identical symbols. Note: To avoid confusion of curves, one curve has been off-set to the right, and raised, and additional time and entry scales relating only to this curve have been inset. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

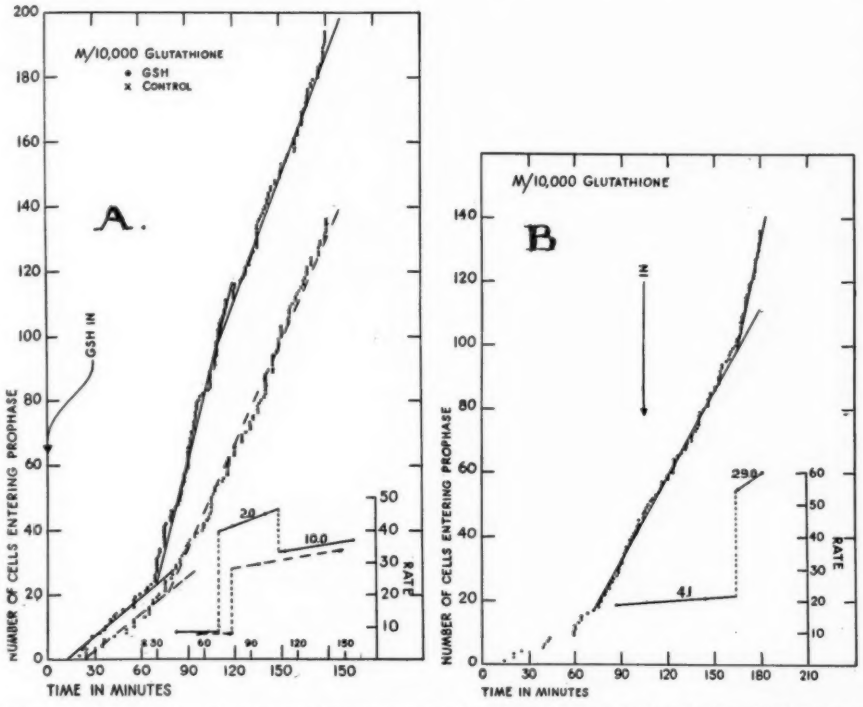

FIGURE 7.—The number of cells entering prophase as a function of time, and the effect on this function of addition of GSH to the environmental fluid. Corresponding curves for rate and entry are shown by identical symbols. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

the amino acids probably appear and act only as components of more complex molecules. For convenience the reduced glutathione [4] will be designated here as GSH.

In the first two experiments GSH at a concentration of M/10,000 was used, and a simultaneous control experiment was performed. The cells for the series, after selection from culture, were washed in one dish and then distributed, as each was removed, alternately to the experimental and control dishes, thus obviating any subjective element in selection as far as possible. In the first experiment the GSH was added at the beginning of the experiment. The resultant curves are shown in figure 7. It is quite evident that a very marked increase in rate of entry into mitosis supervened in the GSH solution 1 hour after the beginning of the experiment. Since this period covered the period of adjustment, it was thought possible that the effect might be thereby modified, and hence a second trial was made in which the GSH was added 105 minutes after the beginning of the trial. The result is also given in figure 7. This

[4] *It was found impractical to work with oxidized glutathione because (1) it could not be obtained in pure state, and (2) it broke down under the conditions required for the experiment giving up some N as NH_3, as was shown by test with Nessler's reagent.*

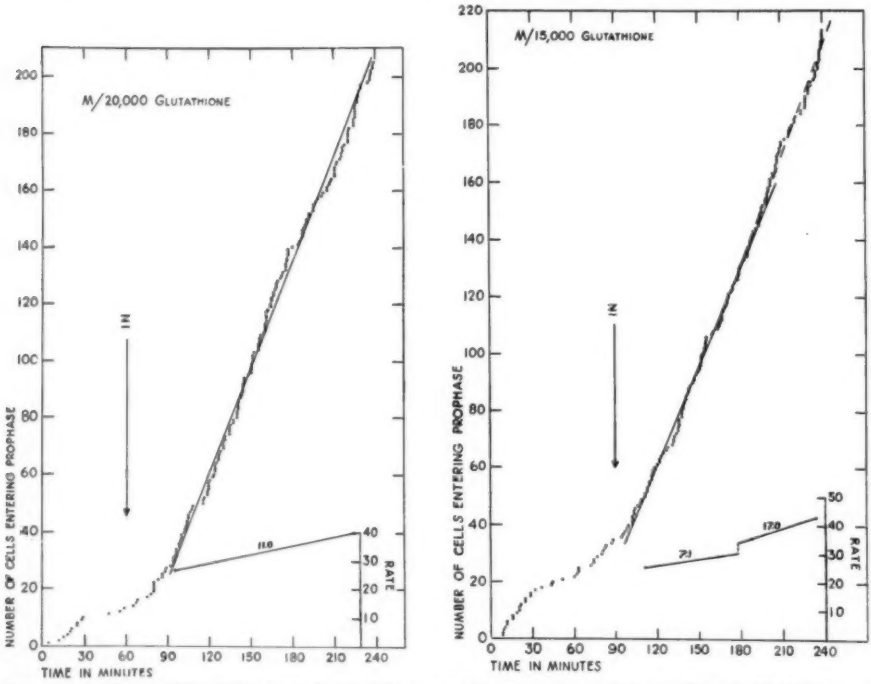

FIGURE 8.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of GSH to the environmental fluid. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

shows again that approximately 1 hour after the admission of the GSH the rate of entry was suddenly increased. The stimulation of rate is of the same order as the entire rise in the GSH curve in the first experiment. It is judged from this that the stimulation is not additive to the rise normally occurring in the process of adjustment; the value for stimulation by GSH in the first experiment should not, therefore, be taken as the difference between the recovery rise in the control and that in the GSH curve, but simply as the full rise shown in the GSH.

After this a series of experiments were performed using molar concentrations of GSH as follows: 50×10^{-6}, 65×10^{-6}, 82×10^{-6}, 135×10^{-6}, and 200×10^{-6} (i. e., 1/20,000, 1/15,000, 1/12,500, 1/7,500, 1/5,000 mols per liter). The results are presented in figures 8 to 10, inclusive. Figure 11 shows the duration of the latent period, the amount of rate at stimulation, the ratio of this stimulation to the extent of the depression on recovery, the acceleration after stimulation and its duration, and the acceleration after recovery as functions of the concentration of GSH in the environment. While stimulation, acceleration after stimulation, and the ratio of recovery to stimulation all have optimum values when the concentration of GSH is 100×10^{-6} mols, the latent period, the duration of the high acceleration following stimulation, and the degree of acceleration after recovery do not; the duration of the latent

period falls, while the other two quantities increase in value with increasing concentration over the range studied.

With respect to the extent of the stimulation and acceleration occurring, if all rates after stimulation are averaged, the value is 41.9 with a standard deviation of 5.8; the average rate at stimulation is 22.4 with a standard deviation of 5.8, a difference in the average amount of stimulation of 19.5 with a standard deviation of 3.2. The difference between the average acceleration just preceding and the average acceleration immediately following stimulation is 16.9, with a standard deviation of 1.5. These figures show that the average rate was raised in stimulation by a factor of 1.87, and the average acceleration was increased by a factor of 5.08. There can be no question as to the significance of such figures.

Effect of a Mixture of the Components of GSH

It seemed of interest to compare the action of GSH at its most effective concentration with the action of a mixture of its component amino acids in equimolar concentration. An experiment similar to the initial one performed with GSH was set up, but instead of using a control solution and one made M/10,000 for GSH, a solution made M/10,000 for each of three

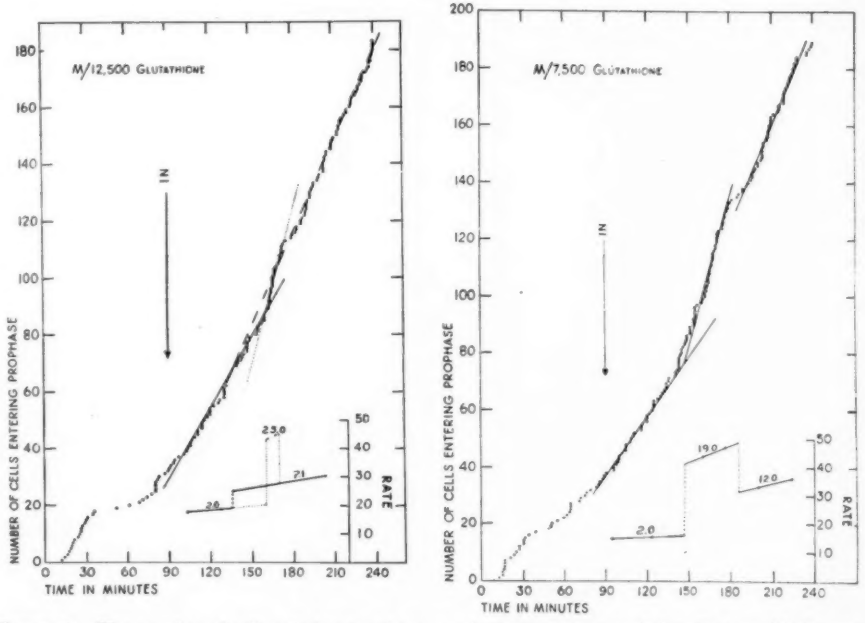

FIGURE 9.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of GSH to the environmental fluid. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals. Note: The dotted part of the rate curve shows the march of the rate, if the dotted part of the entry curve is considered to hold. The more simple curve is that derived from the entry curve, if the dashed time is used. The march indicated by the dotted line is considered more probably true in view of the march of the curves at higher concentrations of GSH.

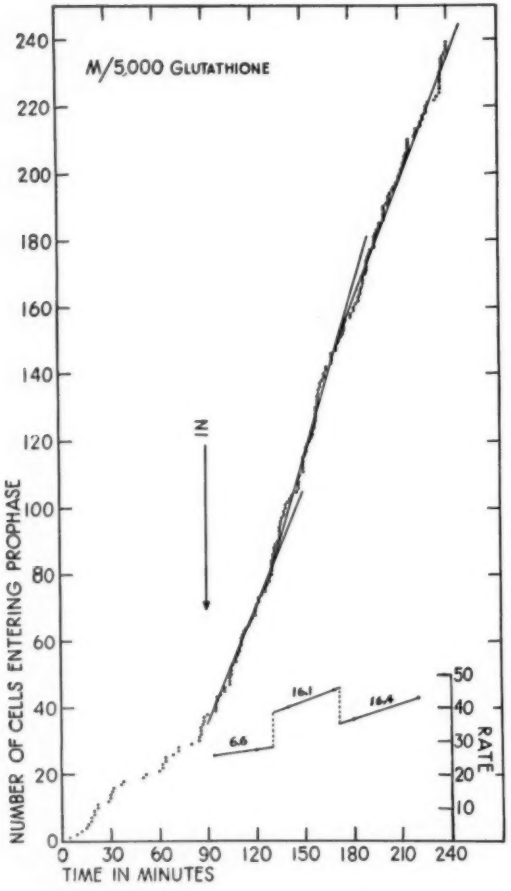

FIGURE 10.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of GSH to the environmental fluid. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

components, glycine, cysteine, and glutamic acid, and one made M/10,000 for each of two components, glycine and glutamic acid, were used as the reagents. The results are given in figure 12. It is at once apparent that the curve obtained from the three components mixed together exhibits the characteristics of those obtained with M/10,000 cysteine, while with the two-component mixture a single slight stimulation obtains. In the three-component curve, the final stimulation observed with cysteine is markedly present, the subsequent acceleration is quantitatively equivalent to that obtained therewith, and the period of depression appears possibly somewhat curtailed.

EFFECT OF CYSTINE DISULFOXIDE

Hammett asserted that in solutions of cystine disulfoxide, cell division is inhibited. It was of considerable interest, therefore, to ascertain the action of this compound.

Three experiments (fig. 13) were performed. In the first two (fig. 13, *A*)

the solution, M/10,000 in concentration, was made up at about 8:45 a. m. and used throughout the day, a fresh lot being made each day. In the last (fig. 13, *B*), a double trial was made in a way similar to that employed in the initial experiments with GSH; in one trial the solution was made fresh just before adding so that the amoebae were exposed to a solution zero to 2 hours old; in the other, the solution was made up just before the test so that the solution was 2 hours old when added, and the amoebae were exposed to solutions 2 to 4 hours old. This was done because Lavine (7) found that the disulfoxide is very unstable in solution at pH 7.0, and all experiments were made at pH 6.8 to 7.0. The results speak for themselves. The fresh solution had a negligible effect while the older solution was strongly depressing for a period of 8 minutes; then there was a recovery of such magnitude in the first experiment and in the one in which the "old" solution was used, as to suggest stimulation. The two initial trials, it will be seen, follow the course of the old solution giving a good check on this result.

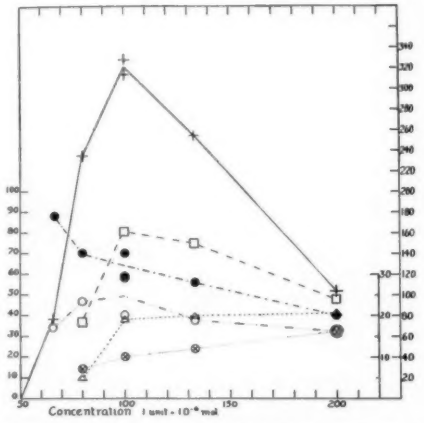

FIGURE 11.—Certain variables of the process of fission in *Amoeba proteus* as functions of the concentration of GSH in the surrounding fluid. Abscissas, concentration of GSH in mols $\times 10^{-6}$. Ordinates: left-hand scale, time in minutes; right-hand full scale, change in rate $\times 10^{-5}$, and ratio of change in rate at stimulation to change in rate at recovery $\times 100$; right-hand partial scale, acceleration. The variables presented are as follows: (●) The duration of the latent period; (△) the duration of the stimulation period. (Read ordinate values for these on the left-hand scale.) (+) Change of rate at stimulation; (□) ratio of change in rate at stimulation to change in rate at recovery. (Read ordinate values on right-hand full scale.) (○) Acceleration during stimulation period; (⊗) final acceleration. (Read these on inset scale at right.) Note that the duration of the latent period, the duration of the stimulation period, and the final acceleration do not show a maximum at about 100×10^{-6} mols GSH concentration, while the other variables do.

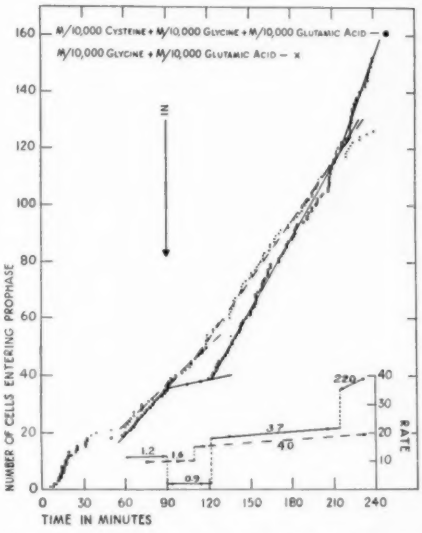

FIGURE 12.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of (1) cysteine plus glycine plus glutamic acid, and (2) glycine plus glutamic acid, to the environmental fluid. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

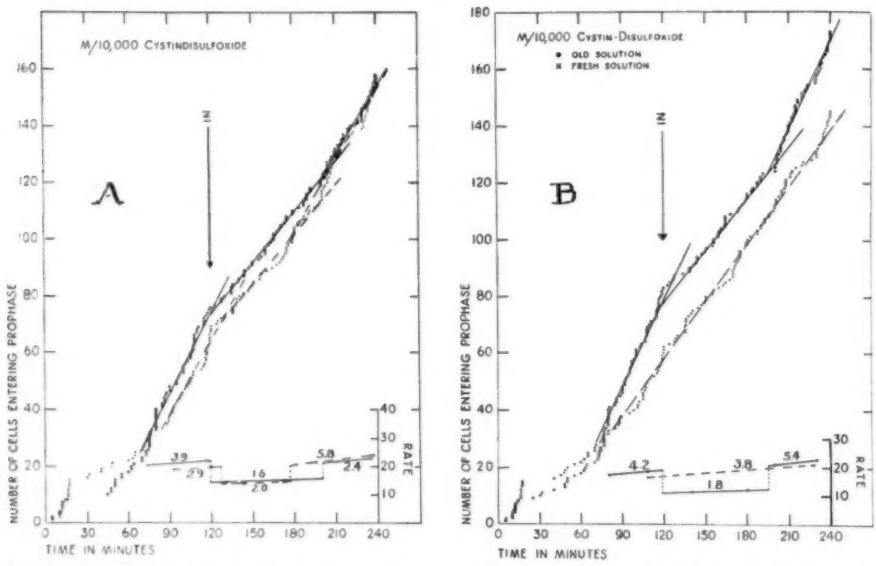

FIGURE 13.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of cystine disulfoxide to the environmental fluid. *A*, Two experiments using solutions made up at the beginning, and allowed to age throughout; *B*, Two experiments, one with a solution freshly made for each test, and one with a solution aged 2 to 4 hours before use. Total number of cells used in *B:* old solution, 608; fresh solution, 448. Rate unit is change per cell per minute$\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

EFFECT OF CYSTEIC ACID (SODIUM SALT)

One of the end breakdown products of cystine disulfoxide in solution, according to Lavine (*7*), is cysteic acid, and the other is cystine. The effect of cystine having already been ascertained, it seemed wise to obtain data on the effect of the other end product. The acid was used in the form of the sodium salt since a pH value of 6.8 to 7.0 had to be maintained. The result is presented in figure 14. There is, it will be noted, a suggestion of a depression effect similar to that obtained with cysteine, which, as it is so slight, may be attributed to at most a slight shock effect. This seemed to be borne out qualitatively during the experiment when some quiescence was noted in the cells just after the addition of the reagent. It is not statistically significant, so the result must be considered negative.

ORGANIC COMPOUNDS NOT NORMALLY OCCURRING IN ORGANISMS

To investigate a large series of organic substances containing —SS or —SH groups would entail an expenditure of time at present unwarranted, but it seemed of interest to test some that were available.

The compounds were chosen to obtain at least a tentative idea as to the dependence of the action of the types of compounds used on their molecular configuration apart from the probably active sulfur-containing groups. The compounds were the dipeptides, diglycylcystine and cystinyldiglycine, in order to ascertain the effect of the —SS linkage to the carboxyl or

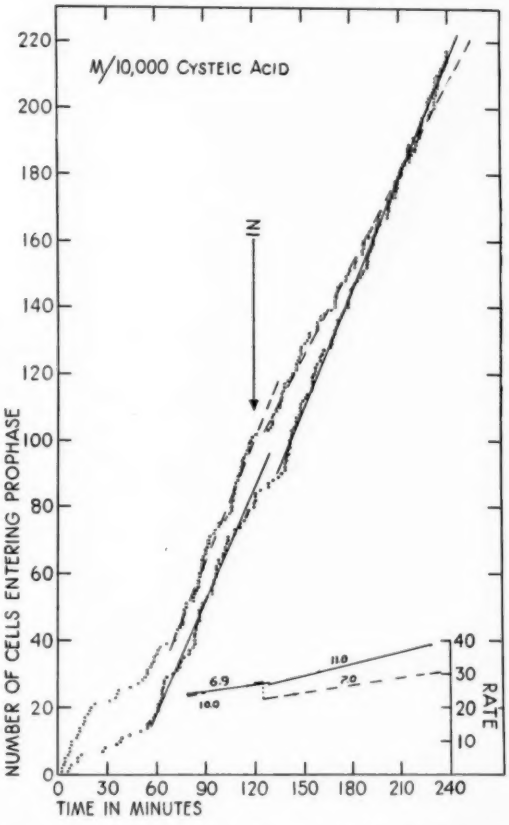

FIGURE 14.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of cysteic acid to the environmental fluid. Two experiments. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

amino groups in isomeric compounds, and β, β'-dithiodipropionic acid, in order to test the effect of the absence of the amino groups from the cystine molecule. These compounds were of the highest purity, the dipeptides being preparations used in measuring their physical constants, and the cysteic acid having been prepared with extreme care and recrystallized seven times.

EFFECT OF DIGLYCYLCYSTINE

Diglycylcystine was used in M/10,000 concentration. Two experiments were made, and the results are shown in figure 15. After a latent period of from 47 to 60 minutes, a definite stimulative effect could be noted. In one test this was followed by recovery so that the rate curve obtained is very similar to that obtained with GSH. In the other the latent period was very long, 65 minutes, and no recovery was observed.

EFFECT OF CYSTINYLDIGLYCINE

Two experiments were performed using cystinyldiglycine in M/10,000 concentration. The resulting curves (fig. 16) show that the addition of this compound resulted in an immediate depression of the rate of entry.

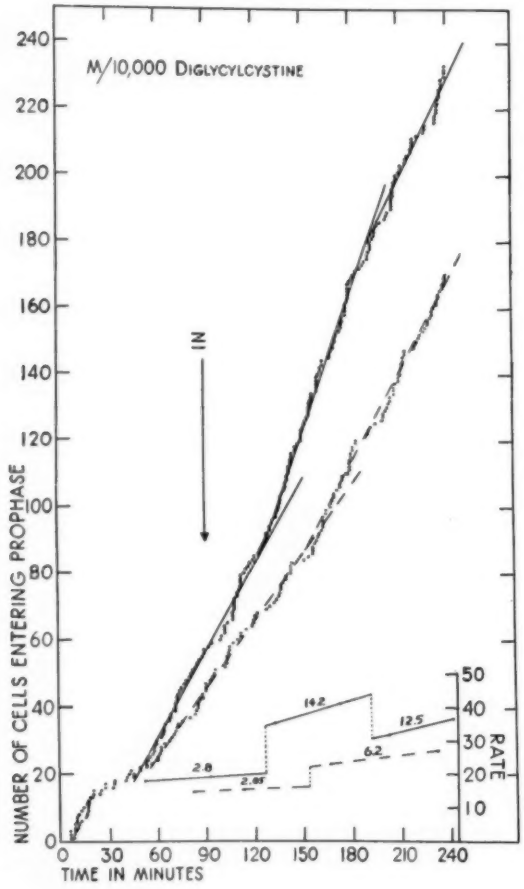

FIGURE 15.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of diglycylcystine to the environmental fluid. Two experiments. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

EFFECT OF β, β′-DITHIODIPROPIONIC ACID

β, β′-dithiodipropionic acid was found to be decidedly unstable in neutral solution. Therefore an experiment was run using solutions brought to the required hydrogen-ion concentration (pH 6.8 to 7.0) immediately before using. The test shows some inhibitive effect (fig. 17).

DISCUSSION

Leaving out the question of the adjustment period, the curves obtained in these experiments fall into three classes: (1) Those showing negative effect; (2) those showing an initial stimulation effect; and (3) those showing an initial depressive effect. In the negative class, are the controls including the one run in the culture medium, the curves obtained with methionine, the curves of concentrations of GSH so low as to show no effect, and the curve obtained with a fresh solution of cystine disulfoxide. The negative result with methionine and the cystine disulfoxide may be taken to indicate, as might well be

expected from Hammett's results, that a high degree of lability of the sulfur group in these compounds is necessary if they are to have immediate action.

In the second class (showing initial stimulation), the curves obtained with cystine, and the mixture of glycine and glutamic acid both show a single stimulation phase of moderate value, followed by an acceleration phase of higher value than that initially obtained. Those with GSH and diglycylcystine show an initial stimulation phase of moderate or high value, succeeded by an acceleration phase as before (in GSH this acceleration is very rapid), and after this a depression, or recovery phase, which is followed by another accel-

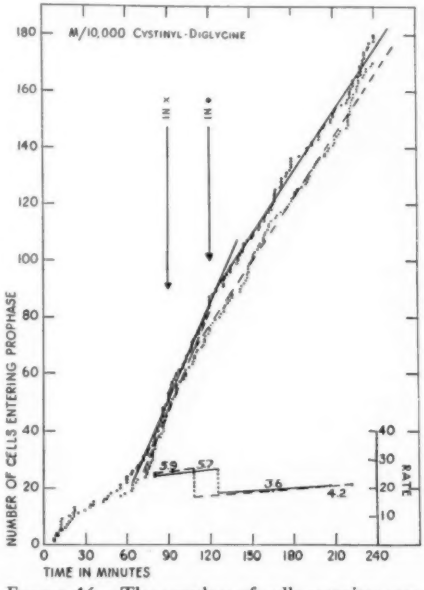

FIGURE 16.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of cystinyldiglycine to the environmental fluid. Two experiments. Rate unit is change per cell per minute $\times 10^4$ acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

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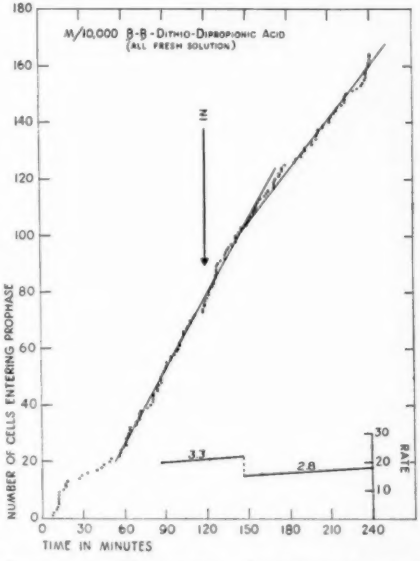

FIGURE 17.—The number of cells entering prophase as a function of time, and the effect on this function of the addition of β, β'-dithiodipropionic acid to the environmental fluid. Rate unit is change per cell per minute $\times 10^4$; acceleration (printed on the rate curves) is change in rate in such units over 100-minute intervals.

eration phase less in magnitude than that immediately following the stimulation, but greater than that preceding it.

In the third class (showing initial depression), the curves obtained with cystinyldiglycine and β, β'-dithiodipropionic acid show a depression phase followed by an acceleration phase, the magnitude of this last in every curve being less than that of the initial acceleration, i. e., that preceding the addition of the reagent. The curves obtained with the old solutions of cystine disulfoxide show a moderate depression followed by an acceleration phase less in magnitude than the initial phase, then a stimulation or recovery phase equal, or approximately equal, to the initial depression (the values do not differ significantly); this in turn is followed by an acceleration

phase approximately equal in value to the initial one, i. e., a transitory depression with full recovery and restoration of initial acceleration. The curves with cysteine are the most complex obtained. All, including the curve obtained with the mixture of glutamic acid, glycine, and cysteine, show that the phases occur as follows: a depression, an acceleration phase of low value, a stimulation exceeding the depression, an acceleration well above the initial value, a stimulation, and lastly an acceleration of a high value. Thus, following depression, the typical curve exhibits first recovery, and finally a high stimulation. In addition there are the curves obtained with cysteic acid. These are shown as exhibiting very slight depressions. They are statistically insignificant depressions, and the accelerations that follow are also insignificant statistically. It is, however, suggested that the breaks are quite similar to those in the cysteine curves. The curves must, however, be considered at present as negative.

At this point it is well to emphasize that no complete inhibition is shown in these curves. All depressions are transitory. Particularly, attention must be called to the fact that in all experiments the final acceleration was positive. Thus, while a lower rate obtained at the end of an experiment than existed initially, it was an accelerating rate.

The data show the following relations between curves: Cystine curves (fig. 4) exhibit definite stimulation and increased accelerations. The curve obtained with β, β'-dithiodipropionic acid (fig. 17) shows depression and decreased acceleration. It is concluded that the NH_2 groups, absent in the last named acid, are essential for the stimulation effect of cystine. Diglycylcystine (fig. 15) is stimulative in action, while the isomer, cystinyldiglycine (fig. 16), is depressive. In this instance, the conclusion must be drawn that even when amino and disulfide groups are present, the configuration of the carrier is of importance since it obviously can lead to reversal of action. Cystine disulfoxide (fig. 13) when used freshly made gave negative results. If a solution some hours old was used, however, a pattern of response showing depressions and recovery with slight stimulation and a final increase in acceleration was obtained. Lavine (7) reports that in alkaline or neutral media, the disulfoxide rapidly breaks down to cystine and sulfinic acid, and the latter in turn is somewhat more slowly oxidized to the sulfonic form, cysteic acid. The data above presented show that cystine has a stimulative effect and that cysteic acid is negative. The curve for the old sulfoxide may be tentatively interpreted, if it is assumed that the sulfinic acid is depressing in action, and more effective than cystine. If this be assumed, the breakdown of the sulfoxide releasing the sulfinic form and cystine first produces depression. This acid, however, is oxidized, leaving cystine, which stimulates, and cysteic acid, which has no action, hence the depression is followed by recovery and finally some stimulation. Against this somewhat facile explanation is made the point that it does not explain the step-by-step response actually observed, as one would expect much slower, or at least less discontinuous, changes in rate. If accepted as a hypothesis, it must be taken as only a partial one.

To turn to the quantitative side of the data, an examination of figures 5 and 7 to 12, inclusive, will show that the highest acceleration obtained with GSH in concentration from M/15,000 to M/7,500 is close to the highest acceleration obtained with M/12,500 and M/10,000 cysteine (including the curve obtained with a mixture with glycine and glutamic acid). The averages of these two groups of accel-

erations are, in fact, 21.7 and 22.0, with standard deviations of 4.2 and 1.8, respectively. This was noted; it was also noted that the rates at the beginning of those high acceleration phases averaged 44.72 and 41.75, with standard deviations of 4.1 and 4.8, respectively. The initial rates in these two sets of observations at the time when the first reaction appeared averaged 18.7 and 18.1, with respective standard deviations of 3.8 and 3.1. Thus the highest rate, the net difference between that rate and the initial rate, and the acceleration of the phase succeeding the attainment of the highest rate are identical within experimental limits. The GSH curves show that the attainment of this peak of rate and acceleration was succeeded by a recovery and a depression phase. In the cysteine curves, this last was not observed but the experiments were all terminated before such a recovery could be expected. In the opinion of the writer such a course for the curve seems quite probable. Allowing for this, the quantitative end results for stimulative and accelerative effects are equal in both curves. They differ in the time required to attain these high levels and in the path taken. With GSH, the result is attained in a single stimulative phase; with cysteine, by a very complex pattern of depression, acceleration, and stimulation phases. While any interpretation must be speculative, it seems permissible to draw the inference that this pattern of similarity and difference means that a single, or at least similar, physiologic state is attained with both reagents, but through a more complex metabolic process with cysteine than with GSH. With respect to cystine, the net average stimulation with GSH, i. e., the average difference between the rate immediately after the recovery phase and the rate just prior to the stimulation phase, is 11.0; for the cystine curves (fig. 4) the total stimulation is 10.4. Also the average acceleration after recovery in GSH approximates in magnitude the average of that ensuing after stimulation with cystine (8.2 and 7.2, respectively). In this instance, if the acceleration phase following the first stimulation in GSH is omitted, the net result of cystine and GSH would be very close. In fact, the data suggest (but by no means demonstrate) that quantitative similarities exist between the respective patterns of response that they elicit, which may well imply correspondingly similar patterns of metabolism.

To turn now to a more general survey, the data show quite clearly that the entry of the adult amoeba into mitosis can be hastened or retarded by relatively slight modifications of the environment. Of the reagents used and studied, those containing sulfur in labile form, —SH–, —SS–, were very effective. In general these were stimulative in action, but it must be noted that the initial action of cysteine is depressive, and that β, β'-dithiodipropionic acid and cystinyldiglycine depressed within the time covered by the experiment. The response observed cannot, therefore, be attributed to the sulfur grouping alone.

A marked characteristic of the curves is that they all exhibit regions of rapid change in rate and regions of slow change, resulting in a steplike appearance of the rate curves. Such curves show definitely that the processes set in action by a relatively simple reagent such as cysteine must be complex. Another characteristic is the occurrence in several curves of actions in opposite sense arising under the same impulse (i. e., recovery phases). Thus, with GSH, stimulation is followed by depression; the same is true for diglycylcystine. With both cysteine and disulfoxide, depression is followed by stimulation. Also in one instance, with cysteine,

occurs the phenomenon of depression, followed by a stimulative recovery, and then a further stimulation. In almost all instances, the acceleration phases that follow change in a corresponding fashion and tend to be of constant value until a new phase of rapid change occurs. Such response suggests that metabolic equilibria within the cell are so disturbed that reaction results in the establishment of new equilibria.

The latent period in the cases studied varies inversely with concentration. This most probably represents the effect of the concentration of the reagent on the rate of its entry into the cell. The extent of the initial stimulative effect, however, especially in the case of GSH, is obviously not a simple function of this rate (fig. 11). In the particular case of GSH, there are, as previously pointed out, two distinct classes of quantitative results: (1) those that pass through an optimum at about M/10,000, stimulation, ratio of stimulation to recovery, and acceleration after stimulation; and (2) those that do not, but are either inverse (latent period), or direct (final acceleration) functions of concentration.

It is necessary, therefore, to posit at least two factors involved, whether one considers the stepwise responses or the effect of concentration in the production of these effects. We are, therefore, barred from ascribing the action on mitosis to a single component, or active group, per se.

In this respect, it must be stressed that the relation of these results to those obtained by Hammett and his collaborators or to previous results reported from this laboratory is not yet clear.[5] For, as pointed out in the introduction, here the matter is solely the rate of entry into mitosis while in much other work, under the term "rate of fission" has been blanketed not only this rate, but also the velocity of cytoplasmic and nuclear fission, and also, in most instances, so large a part of the growth period as to make it necessary to question the part growth played. Even here the question might be raised. The writer feels it to be most unlikely that a process capable of raising the rate of entry into mitosis by 80 percent in approximately 15 minutes, or in that same space of time increasing the acceleration of that rate by 500 percent in a group of mature cells, is one that will ordinarily be subsumed under the concept of growth, and that to designate such response as stimulation is not a misnomer.

It seems apparent from these results that the processes underlying the entry into mitosis depend on the fulfillment of a complex set of conditions which are quite labile and easily disturbed.

A careful study of the changes that could be elicited in certain characteristic response patterns by a carefully chosen series of reagents might prove useful for the study of these processes. It would, for instance, be of considerable interest to ascertain if the patterns are changed in cells long-exposed to the action of carcinogens.

SUMMARY

A method is described for the selection, from a culture of amoebae, of groups of cells in which many cells will divide during a short period after selection. Using such cells, a study of the effect of certain reagents, several sulfur-containing organic compounds in particular, upon the initiation of fission was made by measuring the effect of their addition to the environmental fluid in high dilutions (2×10^{-4} to 5×10^{-3} mols per liter) on the number of cells entering prophase as a function of time. Methionine and cysteic

[5] *It is just possible, and this suggestion is advanced with the utmost reserve, that the acceleration phases might be an expression of the effect on nuclear growth such as was found by Chalkley and Voegtlin with GSH.*

acid gave essentially negative results. Cystine, cysteine, glutathione, cystine disulfoxide, β, β′-dithiodipropionic acid, cystinyldiglycine, and a mixture of glutamic acid and glycine all affected the course of the function.

Two classes of effect were obtained: (1) A sudden saltatory change in rate, either an increase or a decrease; (2) a change from one level of constant acceleration to another, again in either direction. The effect of a given reagent is a more or less complex pattern of these two types. In some patterns (cysteine) changes in both directions of both types obtained.

Cystine and β, β′-dithiodipropionic acid gave opposite responses, as did cystinyldiglycine and diglycylcystine.

It is concluded that the mechanism governing the initiation of fission in *Amoeba proteus* is characterized by lability and considerable complexity. Its relation to overall growth processes or to growth of the nucleus is not yet clear. It does not appear that the presence of labile sulfur alone, irrespective of other chemical groups in a given reagent, insures its stimulative action.

It is suggested that it would be of interest to ascertain whether the subjection of cells to carcinogens results in modifications that can be revealed through changes in the response pattern to the reagents used.

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Effect of a Low Cystine Diet on the Growth of Various Strains of Mice[1]

By FLORENCE R. WHITE [2] and JULIUS WHITE, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The desirability of studying the effect of restricted cystine intake on the growth of various types of tumors in mice has led us to devise a diet for the mouse which would meet this requirement.

The ideal diet for this type of study would be one composed entirely of synthetic substances such as purified amino acids, crystalline vitamins, etc. However, two important factors prevent one from employing this type of diet at the present time. In the first place, a completely synthetic diet which is adequate in all respects for the mouse has not yet been devised, and in the second place the expense of a highly synthetic diet makes a study employing large numbers of animals impracticable.

Other investigators employed diets in which the cystine or methionine content of the diet for the mouse could be restricted. Voegtlin, Johnson, and Thompson (*1*) employed a low cystine diet in their studies on the growth of spontaneous mammary tumors in C3H mice; Morris and Voegtlin (*2*) made a similar study in which they employed a diet which was relatively low in methionine. The chief source of protein was arachin.

We endeavored to use readily available ingredients whose cystine (and methionine) content was well-known in devising a diet in which the cystine level could be readily controlled. This paper deals with the results of this study.

Originally, this diet was intended only for short-time growth experiments. However, other problems arose which made it advisable to employ this low cystine diet as well as the cystine-supplemented one over prolonged periods of time. In table 1 is indicated the composition of the low cystine diet. The replacement of 0.5 percent starch by 0.5 percent *l*-cystine constituted the so-called high cystine diet. Mice ingesting the low cystine diet for a period of 12 months or longer had a tendency to have rough coats and breed very poorly but showed no structural changes in the organs or tissues. No permanent stunting was apparent since it was possible for the animals on the low cystine diet to resume normal growth immediately and rapidly upon the addition of cystine to the diet.

These diets were tested on the following strains of mice of both sexes: C3H, ABC, Bagg albino, C, A, dba (subline 212), I, and AK.

TABLE 1.—*Composition of diets*

Ingredients	High cystine diet	Low cystine diet
	Percent	*Percent*
Casein Labco (vitamin-free)	3.9	3.9
Starch	54.6	55.1
Crisco	22.0	22.0
Cod-liver oil	3.0	3.0
Salts (modified Osborne-Mendel)	6.0	6.0
Ryzamin B (Burroughs Wellcome)	5.0	5.0
Liver extract (Lilly 343)	5.0	5.0
l-Cystine	.5	0
Total	100.0	100.0

For our purposes, the mice were almost always placed on these diets at weaning, which, in all cases, was 4 weeks. We also placed older mice up to 8 weeks of age on

[1] *Part of this work was carried out at Yale University Medical School, Department of Physiological Chemistry, and aided by a grant from the Hubbard-McCormick Clinical Cancer Research Fund.*

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these diets. In this latter group, the mice on the low cystine diet would have a tendency to show a slight loss in weight during the first week but after that would maintain their weight or show a slight gain.

Figure 1, *A* and *B* are representative

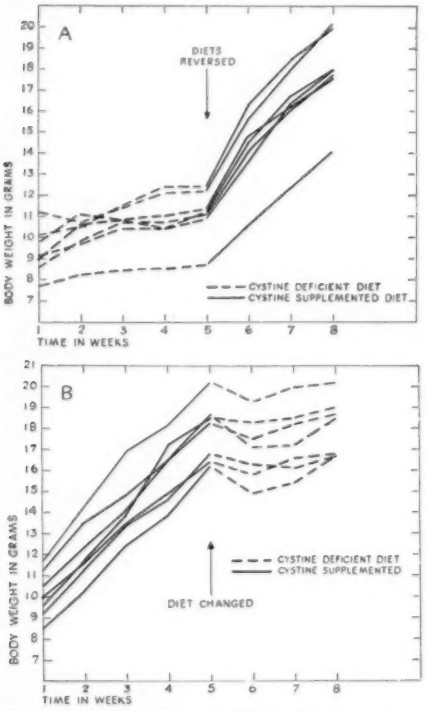

FIGURE 1.—Response of C3H mice to cystine-deficient and cystine-supplemented diets. These curves are representative weight curves of one strain of mouse. All other strains studied showed essentially the same type of response. At least 50 mice were used in each strain studied.

weight curves of one strain of mouse,

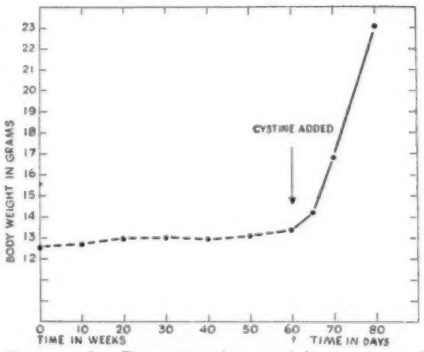

FIGURE 2.—Representative weight curve of animals ingesting the low cystine diet for 60 weeks or more. At 60 weeks, 0.5 percent *l*-cystine was added, and a continuous and rapid increase in weight was observed. This curve is representative of dilute brown mice (subline 212) but is typical of all strains studied.

namely, the C3H. All other strains of mice studied showed essentially the same type of response. The weight curve for animals ingesting the low cystine diet for prolonged periods of time is shown in figure 2. This curve is for the dilute brown mouse (subline 212), but is representative of all strains of mice studied. Similarly, the rapid and continuous increase in weight upon the addition of cystine holds for all strains studied.

These diets have already been employed (*3*) in the study of the effect of restricted dietary cystine on the reaction of dilute brown mice to methylcholanthrene. The use of these diets in studies on the incidence of spontaneous mammary tumors, induced tumors, and spontaneous leukemia in mice will be reported later.

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Extraction and Ultracentrifugation of Mammary Tumor Inciter of Mice

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The existence in the milk of high-mammary-cancer strains of mice of a factor (or factors) associated with the production of mammary-gland cancer has been definitely established (cf. Bittner (*1*) for a review of the literature). Recent reports indicate that the active agent is probably present in other tissues and body fluids besides milk, e. g., lactating mammary gland (*2*), spleen (*2*), thymus (*2*), mammary-gland tumor (*3*), and whole blood (*4*). Studies now in progress at the National Cancer Institute were designed to determine the nature of the agent, hereinafter referred to as the *mammary tumor inciter* (or MTI).[1] The results of some of the preliminary experiments which have reached a stage of definite significance and which have already served as a basis for the planning of further studies are presented at this time.

PRESENCE OF THE MTI IN SALINE EXTRACTS OF MAMMARY-CANCER TISSUE

Bittner (*3*) has shown that the MTI is present in spontaneous-mammary-cancer tissue of mice. In view of the technical difficulty of obtaining the large quantities of milk required for certain studies on the MTI, the possibility of using extracts of tumor tissue as a source of the agent was investigated. The extracts were prepared by grinding freshly excised spontaneous mammary tumors, from C3H females, with sand in a mortar and diluting with 0.9 percent NaCl solution to give a final concentration of 20 percent by volume of tumor tissue. The extracts were cleared of sand and gross tissue particles by centrifugation in an ordinary centrifuge [2] and fed [3] in 0.2- or 0.4-cc. amounts to 46 female mice that were 7 to 14 days of age. The test animals were F_1 hybrids derived by mating strain I (low tumor) females and strain C3H (high tumor) males. Mice of this derivation develop few spontaneous mammary tumors but are highly susceptible to the MTI derived from strain C3H mice.[4]

Three separate tumor extracts, prepared from as many different tumors, were employed. Mammary cancers have resulted in test animals fed each of the three extracts, the incidence being the same, within limits of error, in each case. The combined results for the three materials are shown in table 1, together with the average age of the mice now living without tumors. All of the latter have not had a full opportunity to develop tumors, but the incidence is already so high (45.6 percent) and unquestionably significant, that final conclusions regarding the presence of the MTI in the extract may be drawn. It is apparent from these results that extracts of fresh tumor tissue, prepared as described, may be used as a source of the MTI for experimental purposes.

[1] *This terminology was recently introduced by Woolley, Law, and Little* (4).

[2] *International Centrifuge No. 2, with horizontal head attachment No. 250. The speed was about 2,500 r. p. m., and the time 30 minutes. The centrifuge was installed in a cold room kept at 10° C.*

[3] *Forced feeding was accomplished with a syringe-blunt needle device similar to that described by Morris and Thompson* (5).

[4] *Andervont, H. B.: Unpublished data.*

TABLE 1.—*Induction of mammary cancer in susceptible mice by the feeding of extracts of mouse mammary-cancer tissue*

Group	Total mice	Latent time of tumors (months)							Mice with tumor	Mice living without tumor	Average age of mice without tumor	Average latent time	Tumor incidence
		4	5	6	7	8	9	10					
	Number								*No.*	*No.*	*Months*	*Months*	*Percent*
Mice treated with 20-percent extract of fresh tumor tissue	46	2	....	1	4	4	10	....	21	25	10	7.8	45.6
Litter mate controls	10	....	....	....	....	....	....	....	....	10	10	....	....
Other controls	49	....	....	....	....	....	....	....	....	49	12	....	....

CONCENTRATION OF THE MTI BY ULTRACENTRIFUGATION

Preliminary studies to determine whether the MTI could be sedimented in the ultracentrifuge have been in progress for 13 months. The test animals are C3H females that were born between the hours of 4:00 p. m. one day and 8:30 a. m. the following day, at which time they were removed from their own mothers (high-tumor strain) and given to C57 Black (low-tumor strain) foster mothers to be raised. In view of the possibility that appreciable quantities of milk (containing the MTI) might have been obtained from the high-tumor-strain mothers by the sibs before they were removed, litter mate sisters were used in each of the experimental and control groups of test animals comprising an experiment.

The present study is composed of two similar experiments involving comparable numbers of test animals and performed 3 weeks apart. Since the technical procedures employed and the results obtained are essentially the same in the two experiments, they are described as one and the results of the two are combined for a single analysis (table 2).

TABLE 2.—*Occurrence of mammary cancers among mice fed fractions of ultracentrifuged milk from high-tumor-strain mice*

Treatment	Total mice	Latent time of tumors (months)								Mice with tumor	Mice living without tumor [1]	Average age of mice living without tumor	Average latent time	Tumor incidence
		4	8	9	10	11	12	13	14					
	Number									*Number*	*Number*	*Months*	*Months*	*Percent*
U. C. pellet suspension	17	1	4	1	....	2	6	....	....	14	2	13	9.9	82.3
U. C. supernatant	17	....	....	1	3	1	1	1	....	7	10	13	10.7	41.2
Control	23	....	....	....	....	1	....	....	....	1	21	13	11.0	4.3

[1] 1 mouse in the U. C. pellet group and 1 in the control group died without tumors at the age of 12 months.

Five to six cubic centimeters of milk from several C3H females, obtained by means of an automatic milking device,[5] were pooled and centrifuged in an ordinary centrifuge [6] for the purpose of separating the cream. The separated milk was given a second preliminary run, this time in the ultracentrifuge [7] at 9,000 times gravity for 5 minutes, in order to remove some of the heavy curd material. The latter run also yielded a small amount of cream. The twice-separated milk was finally centrifuged for 1 hour at 60,000 times gravity in the ultracentrifuge.

Two fractions resulted from this procedure: (1) A clear, slightly yellow colored, supernatant fluid; and (2) an opaque, chalky white pellet which was surmounted

[5] *A paper describing this instrument and its use (H. K.) appears in this issue, p. 457.*

[6] *See footnote 2.*

[7] *Air-driven ultracentrifuge of the type described by Beams* (6).

by a thin layer of clear, amber-colored, gelatinous material at its centripetal surface. The supernatant fluid was pipetted off with a capillary tipped pipette, care being taken not to disturb the pellet, and labeled "U. C. supernatant." About 0.5 to 1.0 cc. of the supernatant was left above the pellet in order that the latter would not be disturbed. In one experiment this remaining supernatant was poured off, and the tube was allowed to drain before the addition of buffer solution to the pellet. In the other experiment, however, the supernatant was left in the tube and used with added buffer solution to resuspend the pellet.

The pellet material was resuspended in 2 cc. of phosphate buffer solution (pH 7.0; M/15). The suspension was then diluted to approximately the same volume as that of the original sample of milk employed in the ultracentrifuge tube (4.5 cc.) by the addition of buffer solution. The volume of the pellet was estimated to be about 1 cc., and it was therefore diluted about 4.5 times in order to obtain a U. C. pellet suspension comparable with the original sample of milk with respect to sedimentable material. The fractions were fed [8] in 0.2-cc. quantities to the test animals. A total of 17 mice were used in testing each fraction, and 23 were observed as controls. The results are summarized in table 2. Only 1 tumor has thus far appeared among the controls, while the incidence of tumors in the groups treated with U. C. pellet suspension and U. C. supernatant is 82.3 and 41.2 percent, respectively. This difference is statistically significant ($\chi^2=6.1$; $P<0.02$). The difference in tumor incidence between supernatant and control groups, i. e., 41.2 and 4.3 percent, respectively, is also significant ($\chi^2=8.2$; $P<0.01$).

[8] *See footnote 3.*

Since 41.2 percent of the mice which received the U. C. supernatant developed tumors, it is obvious that some MTI remained in this fraction and that centrifugation at 60,000 times gravity for 1 hour did not give complete sedimentation. That the significantly greater incidence of tumors in the group treated with the U. C. pellet suspension represents a greater concentration of the MTI in this fraction is confirmed by the quantitative relationship between dose and response. Results have been presented (*7*) which show a significant correlation of the incidence of tumors with the number of hours that test animals are allowed to remain with high-tumor-strain females and thus indirectly, with the amount of milk consumed. The mean latent periods were also shown to follow a definite trend, but individual variations were great and the correlation is of less significance.

Additional evidence pointing toward the same conclusions is apparent in other experiments now in progress in this laboratory (H. B. A.). In these experiments, accurately measured quantities of C3H milk were fed [9] to C3H test animals of the type described above. Figure 1 shows the provisional relationship between the amount of milk (in log cubic centimeters) and the respective biologic responses (tumor incidence in probits (*8*) and average latent period in months) as they stand 14 months after the beginning of the experiment. Provisional estimates of the potency relationship between U. C. pellet suspension and U. C. supernatant materials, as estimated graphically from the response curves of figure 1, indicate that 79 to 96 percent of the MTI was present in the pellet suspension whereas only 4 to 21 percent remained in the supernatant. Although these values are not considered

[9] *See footnote 3.*

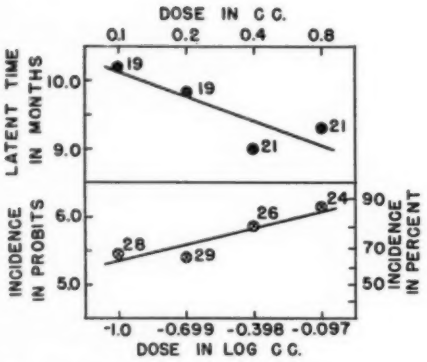

FIGURE 1.—Provisional representation of the relationship between the amount of milk fed and the tumor response in test animals (data of H. B. A., see text).

Crossed circles: Tumor incidence in probits plotted against amount of milk in log cubic centimeters.

Solid circles: Average latent time in months plotted against amount of milk in log cubic centimeters.

Numerals adjacent to plotted points represent the number of animals involved in each estimate.

The right and upper scales give equivalent values in percent and arithmetic dose, respectively.

as precise, there can be no doubt that the potency of the pellet suspension was greater than that of the supernatant and that the MTI was concentrated in the pellet by ultracentrifugation.

DISCUSSION

Although the cream fraction separated from the milk by ordinary centrifugation was not tested in these experiments, nevertheless the possibility can be ruled out that the MTI is present only in the cream.

The suggestion has already been made that the MTI may belong to the group of disease-producing entities classified as viruses (*3, 9*). The fact that the agent was not completely sedimented by a centrifugal force of 60,000 times gravity applied for 1 hour indicates that the size of the active unit particle is less than that of some of the larger known viruses, e. g., vaccinia virus which is sedimented with a much smaller force of 800 times gravity (*10*). This finding also shows that mouse mammary cancer can be transmitted by cell-free material since no cells could have been present in the supernatant after this treatment. The present findings do not constitute proof that the agent concerned is a virus. Nevertheless, they are compatible with the behavior of many viruses.

SUMMARY

Tumors were induced in susceptible mice by the feeding of NaCl solution extracts of fresh tissue derived from spontaneous mammary cancers of mice.

The tumor-inducing agent was sedimented from mouse milk, though not completely,* during a 1-hour period at 60,000 times gravity in an ultracentrifuge.

*ADDENDUM: After this article was submitted for publication, Visscher, Green, and Bittner (Proc. Soc. Exper. Biol. & Med., 49:94–96 (1942)) reported results which indicate that sedimentation of the MTI from mouse milk was accomplished by ultracentrifugation at 110,000 × gravity for 1 hour.

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Apparatus for Milking Mice

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On several occasions apparatus for milking laboratory animals as small as rats has been described (*1–3*). Mice are more difficult to handle in this respect. The present note describes a procedure which has been used successfully in this laboratory and in which the experience of the workers cited has been found to be of considerable value.

The assembly is shown in figure 1. With the plunger in the down position so that inlet *A* is closed, the house vacuum line draws mercury up in the closed electrode tube until contact is made with the upper steel wire, *P II;* meanwhile suction is put on the teat cup *C*. Full voltage then is impressed across the solenoid air valve *S* lifting the movable iron plunger so that air enters through the inlet holes at *A*, drilled in the wall of the valve. This releases the suction acting both on the electrode tube *UT* and the teat cup *C*. The mercury falls, but the plunger is held in place since about half voltage across the lower electrode *P I* leaves a holding current in the solenoid sufficient to prevent the dropping of the plunger until this electrode *P I* is uncovered. The circuit is thereby completely broken, the plunger drops, and the cycle starts anew.

A constriction in the U-tube gives a smooth motion of the mercury, free from oscillations. The solenoid valve plunger should be clean, polished, and dry. The voltage used will depend on the characteristics of the solenoid. We use 24 volts and

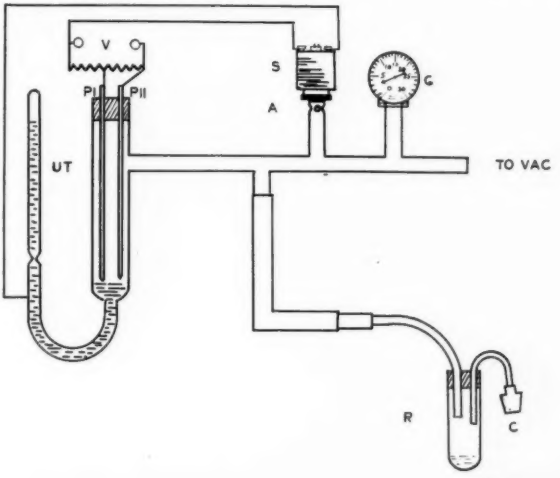

FIGURE 1.—Diagram of apparatus for milking mice.

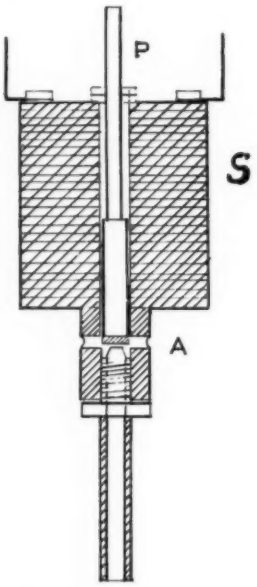

FIGURE 2.—Detail of milking apparatus to show action of plunger, *P*.

a 400-ohm solenoid coil. A small piece of cork is attached to the bottom of the plunger *P*, giving a good seat to the open tip of the tube, required for cutting off the air supply at *A* (fig. 2).

A satisfactory teat cup can be made from an ordinary serum bottle cap. A fairly large flexible opening on the cup is desirable. The suction pulsates from 1 to 7 or 8 inches of mercury at a frequency of 72 cycles per minute.

The breasts of the mice should be moistened with warm water. The lactating mice should be separated from their young in the evening or late afternoon and milked with the machine the following morning. About 0.7 cc. per milking is obtained from average to good mice. The optimum time in the lactation period is from 10 to 20 days. To speed up the yield, three or four teat cups *C* and collection bottles *R* are used simultaneously, by as many operators each with a separate mouse.

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Production of Gastric Lesions in Rats by Fasting, Partial Inanition, and Deficiency of Certain Dietary Constituents

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INTRODUCTION

The development of gastric lesions in rats by several experimental procedures has attracted the attention of many investigators during the last quarter of a century. The lesions produced have been of two types, papillomas of the forestomach and superficial ulcers of the glandular portion of the stomach. The etiologic factors presumably involved have varied considerably; one of significant interest has been the purported relation of certain dietary deficiencies to the genesis of these lesions. It seems desirable to know the exact nature of the specific dietary factors, if such exist, in order to determine whether it would be possible to maintain animals on such deficient regimens long enough for the papillomas to become malignant neoplasms and for the superficial ulcers to become chronic ulcers or even carcinomas. The present paper deals with our attempts to investigate the nutritional aspects of this problem, with a histopathologic analysis of all material.

Gastric lesions in rats were first described in 1913 by Singer (*1*) who believed them infectious in origin. The development of gastric neoplasms in rats infested with the nematode *Spiroptera neoplastica* (*Gongylonema neoplasticum*) was reported by Fibiger (*2–3*). This nematode may not have been the sole etiologic factor responsible for the development of these gastric neoplasms since the animals were fed only white bread, a diet considered inadequate in several nutritive factors. Bullock and Rohdenburg (*4*) considered the lesions of the forestomach described by Fibiger to be the result of nonspecific irritation. They devised several ingenious contrivances for mechanical irritation of the stomach to test this hypothesis; and while they were unable to duplicate Fibiger's results exactly, they did obtain a close resemblance to them.

Passey, Leese, and Knox (*5*) repeated Fibiger's experiments. In the group of rats fed Fibiger's diet of white bread, a few lesions occurred in the forestomach but they were not so severe as in animals on the same diet infected with *Gongylonema neoplasticum*. When the rats were fed a full diet, these investigators found insignificant mucosal changes in the forestomach. Passey (cited by Cramer (*15*)) was unable "to reproduce either the gross papillomata shown in Fibiger's illustrations and that the benign hyperplasia, although comparable with that figured by Fibiger was not of the same degree."

It was not until Pappenheimer and Larimore (*6–7*), in 1923, showed a possible relation of the lesions of the forestomach to a dietary deficiency that the diet was suspected of playing a role in the genesis of these lesions. These investigators did not discover the exact nature of the deficiency. Their diet, No. 84, contained 95 percent patent flour, and mineral salts consisting of calcium lactate, 2.9 percent; sodium chloride, 2.0 percent; and ferric

citrate, 0.1 percent. It was a rachitic diet, deficient not only in the character of the proteins but also in vitamin A, minerals, and probably several vitamins of the so-called B complex. Subsequently Howes and Vivier (*8*), Hoelzel and DaCosta (*9–10*), Sharpless (*11–14*), and others have used white wheat-flour diets modified in various ways and have reported the prevention of the lesions by such substances as yeast, protein, cystine, choline, and specific vitamins. Hoelzel and DaCosta (*10*) reported that, next to fasting, protein deprivation was the most effective means of producing the lesions. As they did not give the composition of their diets, it is difficult to review critically their results.

Cramer (*15*), in 1937, reported two series of experiments conducted 10 years apart, according to his statement under apparently identical conditions, which gave entirely discordant results. In both series of experiments, the animals were fed a vitamin A deficient diet. Different strains of rats were used in the two series, and no information was given on the possible stores of vitamin A in the two series of animals prior to placing them on the deficient diet. Cramer concluded that diets deficient in vitamin A may play a contributory part in the development of papillomatosis but that vitamin A is not the chief determining factor.

The Japanese investigators, Fujimaki et al. (*16*), in 1928, studied the relation of unbalanced diets containing up to 100 percent fat upon the morphologic changes in the forestomach of the rat and concluded that these changes and the metabolic disturbance of fatty substance have a close and intimate relationship.

Roffo (*17*) described adenocarcinomas of the glandular portion of the rat's stomach resulting from feeding the animal milk and bread to which was added an equal part of fat heated to 350° C. for 30 minutes. Such a diet would be difficult to feed to an experimental animal and secure adequate food intake, and if the animal failed to eat it in satisfactory amounts secondary inanition effects might result.

The most recent attempt to relate diet to gastric lesions is that of Brunschwig and Rasmussen (*18*), who fed a great variety of unbalanced diets to young rats and concluded (*18, p. 377*) that "malnutrition resulted in multiple pin-point and acute streaked hemorrhagic erosions and ulcerations in the glandular portions of the mucosa and, to a lesser extent, in the rumen of the stomach." From their experimental approach they were unable to separate clearly the effect of starvation from that of unbalanced diets.

GENERAL PROCEDURE AND DIETS

Three-month-old Wistar strain rats produced in the same breeding colony were used throughout these experiments. They were housed in individual wire-cloth-bottom metabolism-type metal cages in a room maintained at a temperature which did not fall below 80° F. during the experimental fasting periods. During the day when the lights were turned on, the temperature usually rose slightly but did not go above 83° as indicated by the recorded temperature of the animal room for that period. Uniform temperature control is essential in fasting experiments where it is desired to prolong the life of the experimental animal. It is also important that the room temperature be kept near or above the *critical* temperature of the experimental animal.

Experiments of Goto (*19*) and Benedict and MacLeod (*20*) indicate that the critical temperature of the fasting rat is near 28° C. (82° F.). It is believed that the temperature of the animal room in the experiments reported herein was never low enough to be any considerable factor

in the survival period of the fasting rats and certainly not of the animals fed, for mere intake of food materially lowers the critical temperature.

The diets used in this series of experiments were divided into two groups. The following tabulation outlines the procedure followed for the experiments on complete and partial inanition. The purpose of fasting the animals prior to the intermittent feeding experiments was to produce papillomas of the forestomach and ulcers of the glandular portion of the stomach and then to study the recovery or failure to recover after feeding an adequate diet at infrequent intervals. The intervals selected and the amount of food fed were purely arbitrary, although it was planned to obtain varying lengths of survival in the different groups upon partial realimentation. The groups on diets 90 and 91 were not fasted before they were placed on these regimens, because the addition of choline alone was a procedure most nearly like fasting of any procedure followed and the addition of glucose to the choline solution (diet 91) was designed to furnish energy to prolong the life of the animal. It was hoped in this way to produce more severe lesions.

Diets used in complete and partial inanition

No. 70, water only.
No. 89,[1] 8 gm. of diet No. 81 [2] once weekly.
No. 90, 325 mg. choline HCl per liter of water, ad libitum.
No. 91, 325 mg. choline HCl per liter of 25 percent aqueous glucose solution, ad libitum.
No. 92,[1] 4 gm. of diet No. 81 [2] three times weekly
No. 93,[1] 8 gm. of diet No. 81[2] twice weekly.

[1] Preceded by a 7-day fast; water available at all times.
[2] See table 1 for the composition of diet No. 81.

The synthetic diets, the composition of which is given in table 1, were planned to be adequate in all nutritive respects except for a single component in each diet. The protein-deficient regimen was thought to be the most important of the dietary series because of the reports in the literature that gastric lesions were induced by protein starvation. The protein-deficient diet No. 82 contained approximately 12.8 mg. of nitrogen in 100 gm. of diet, unaccounted for by the nitrogen content of the known vitamins in the rice-polish concentrate, the only source of unknown nitrogen in the diet. This amount of nitrogen expressed as protein (N x 6.25) would account for 0.8 mg. of protein per gram of diet, which would be equivalent to 0.4 percent of the amount of protein in a normal diet containing 18 percent casein. This is a relatively insignificant quantity of protein, even though we assume that all of this unknown nitrogen is protein. The diet can, therefore, be considered as essentially protein-free.

Diet 78 of the protein-deficient regimen contained 4 percent casein since Sharpless (*12*) had reported that malignant ulcers were prevented by cystine in a diet containing 4 percent casein. Methionine was

TABLE 1.—*Composition of diets*

Constituents	Diet No.				
	78	81	82	88	94 [1]
	Grams	*Grams*	*Grams*	*Grams*	*Grams*
Casein (commercial)	4.0	18.0	0	41.5	18.0
Sucrose	38.2	24.3	42.3	.....	65.3
Salt mixture (McCollum's No. 185 modified as to calcium and rare elements)	4.0	4.0	4.0	4.5	4.5
Lard	50.0	50.0	50.0	50.3	.....
Corn oil fortified with vitamin A ester [2] and calciferol [3]	1.0	1.0	1.0	1.0	.....
Wheat-germ oil	.5	.5	.5	.5	.....
Choline hydrochloride	.2	.2	.2	.2	.2
Ryzamin B	[4] 2.0	[4] 2.0	[4] 2.0	[4] 2.0	[4] 2.0
Glycerol	.....	.....	.....	.....	10.0
Methionine	.1	.....	.....	.....	.....
Total	100.0	100.0	100.0	100.0	100.0

[1] α-tocopherol, 100.0 mg. per 1 kg. of diet, calciferol 0.1 mg. per 1 kg. of diet, and 90 percent β-carotene 10.0 mg. per 1 kg. of diet, were added in solution in ethyl ether to a part of the sucrose and the ether evaporated at room temperature in a stream of air. Merck & Co., Rahway, N. J., furnished the α-tocopherol.
[2] Supplied by Distillation Products, Inc., Rochester, N. Y.
[3] Furnished by Mead Johnson Co., Evansville, Ind.
[4] 4 mg. riboflavin added to each kilogram of diet.

added to the 4 percent casein diet to ascertain if this sulfur-containing amino acid would furnish protection against the formation of gastric lesions. The control diet in this series was identical with diet 82 except that 18 parts of the sucrose were replaced by 18 parts of commercial casein per each 100 parts of diet.

While it was thought that the most significant findings would occur in the fasting, partial inanition, and the protein-free groups, for the sake of completeness, two other series as shown in table 1 were studied, one deficient in carbohydrate represented by diet 88 and the other deficient in fat, diet 94.

Neither of these last two diets could be said to be entirely free of carbohydrate or entirely free of fat, for in the former the ryzamin B probably contains a small amount of carbohydrate while in the latter the commercial casein contains approximately 1 percent of lipoid material, mostly phospholipin.

RESULTS

The effect of complete and partial inanition upon the body weight of rats is illustrated in figure 1. This figure shows very rapid loss in body weight in all the groups. These curves are constructed from the average weight of not less than 20 animals, and in most cases the number of animals per group was 25.

The loss in body weight of the animals receiving only choline (diet 90) in their water was as rapid as for fasting alone, but the animals lived longer. When glucose was added to the choline solution (diet 91) the loss in weight was less rapid. The extra energy furnished by the 25-percent glucose solution which contained choline prolonged the life of the rats considerably more than did fasting with only choline added.

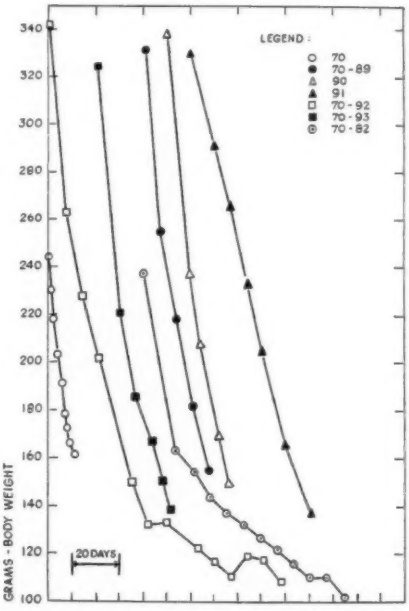

FIGURE 1.—Average body weights of rats: Fasting, diet 70; fed 8 gm. once weekly after fasting 7 days, diet 70–89; choline in drinking water, diet 90; choline in 25-percent aqueous glucose solution, diet 91; fed 4 gm. thrice weekly after fasting 7 days, diet 70–92; fed 8 gm. twice weekly after fasting 7 days, diet 70–93; protein-deficient diet 70–82 after fasting 7 days.

The animals continued to lose weight with one weekly feeding of 8 gm. (diet 70–89) but slightly less rapidly after the addition of food. The feeding of 8 gm. of food twice weekly did not increase the ability of the animal to maintain its body weight, but when the quantity of food offered was decreased to 4 gm. and the frequency of feeding increased to three and, toward the end of the experiment, to four weekly feedings of 4 gm., the animals did not lose weight as rapidly and lived for a much longer period. Within the limits of these experiments the frequency of feeding apparently is more important in preventing body weight loss than the total

quantity of food consumed. The rate of body-weight loss on the diet containing no protein but adequate in other respects (diet 70–82, fig. 1) was not quite so rapid as in the group fed three to four times weekly.

Realimentation following fasting with a normal protein level, consisting of 18 percent casein, is shown in figure 2, diet 70–81. It required nearly 4 weeks on this adequate control diet for the animals to recover the weight lost during the fasting period. The food intake was higher during this recovery period than it was subsequently. The drop in body weight after 60 days was due to the killing of 10 animals for autopsy.

On diet 70–78, containing 4 percent casein after a fasting period, the animals were unable to maintain their body weight but continued to lose weight amounting to 3–4 gm. weekly. The loss was less rapid in this group than in the group on diet 70–82, deficient in protein. The animals on the control diet appeared healthy, and the only abnormal symptom appearing in the other groups was loss in body weight. It is believed the various diets used were adequate in all respects except for the single component under investigation.

In the group fed a carbohydrate-deficient diet (fig. 3), half of the animals were fasted and the other half were started on the diet without prior fasting. The body weight of the former after recovery eventually not only equaled the latter but surpassed them, whereas the animals on the fat-deficient diet (fig. 4) after fasting never again quite equaled the body weight of the animals not fasted but fed the same diet. Both groups increased in body weight, but neither group weighed as much as the animals on the high-fat carbohydrate-deficient diet. There was no difference in food intake between prefasted and non-prefasted animals in either low carbohydrate or low fat groups. The total quantity of food consumed in the groups on diet 94 was considerably greater than that of the animals on the high fat-low carbohydrate groups (diet 88). It is believed this difference is due to the difference in caloric content of the two diets.

The percent survival in the groups completely or partially deficient is illustrated in figure 5. At the top of this figure is given in days the survival of rats when fasting, with choline in the water, and with choline in a 25-percent aqueous solution of glucose. It is interesting that choline about doubled the survival period over fasting and that the addition of glucose considerably increased survival over the addition of choline alone.

The middle bracket of figure 5 shows the survival in the intermittently fed groups. While feeding twice weekly prolonged survival somewhat over feeding once weekly, neither procedure gave as good survival

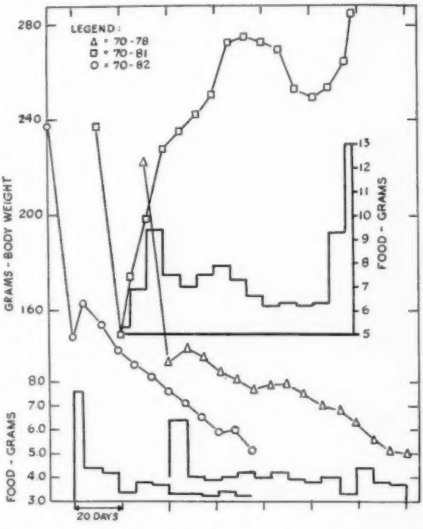

FIGURE 2.—Average body weights and average daily food consumed by rats after fasting 7 days. Diets 70–78, 4 percent casein; 70–81, 18 percent casein; 70–82, no protein.

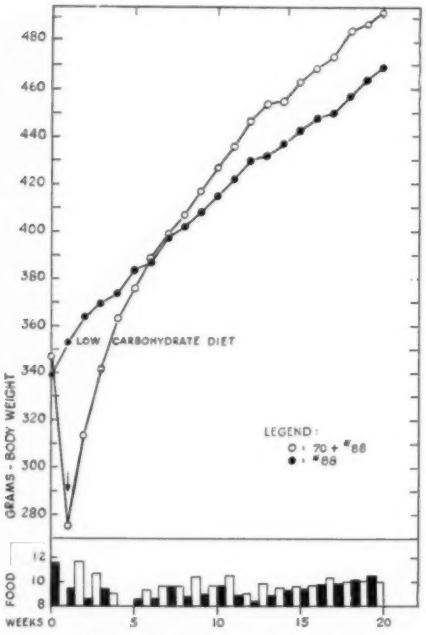

FIGURE 3.—Average body weight and average daily food consumed on a carbohydrate-deficient diet, No. 88; after fasting 7 days, diet 70–88.

as when glucose and choline were given although it must be remembered that the glucose and choline groups were not preceded by the 7-day fasting period.

The feeding of less food (4 gm.) three times weekly very definitely increased the survival period. Some animals lived over 90 days. These animals became very vicious during the last part of their lives and attempted to bite at any object which came near them.

The lower portion of figure 5 shows the survival up to 105 days of the protein-deficient group. Seventy percent of the animals on 4 percent casein which had not been fasted initially were still alive at this time, and less than 20 percent of the animals on 4 percent casein which had been preceded by an initial fasting period, while no animal of the protein-deficient group survived beyond 100 days.

The animal has a remarkable ability to survive on its own tissues following a week of fasting during which 60 percent of its original body weight has been lost.

Table 2 gives the minimum, maximum, and average days which the animals in the various groups lived. This table shows a definite increase in the mean living age from 11.1 days for total fasting to 125.3 days on 4 percent casein not preceded by a fasting period. There is also a definite increase in the maximum length of life in the various groups, but it is also apparent from the data in the minimum survival column that some animals were unable to survive much beyond the fasting period. These survival data are not absolute because most of the animals were killed shortly before impending death in order to have autopsy material unaffected by autolysis. In no case where the animal was found dead was that animal included in the histologic analyses if sufficient autoly-

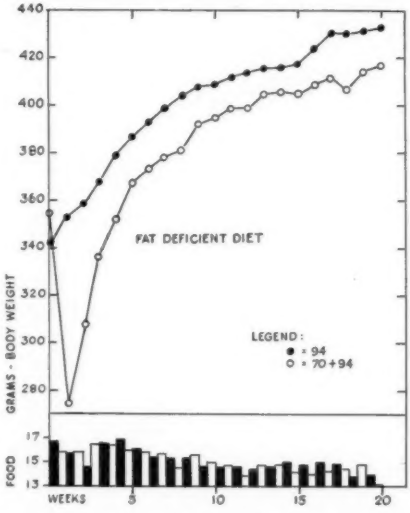

FIGURE 4.—Average body weight and average daily food consumed by rats on a fat-deficient diet 94; after fasting 7 days diet, 70–94.

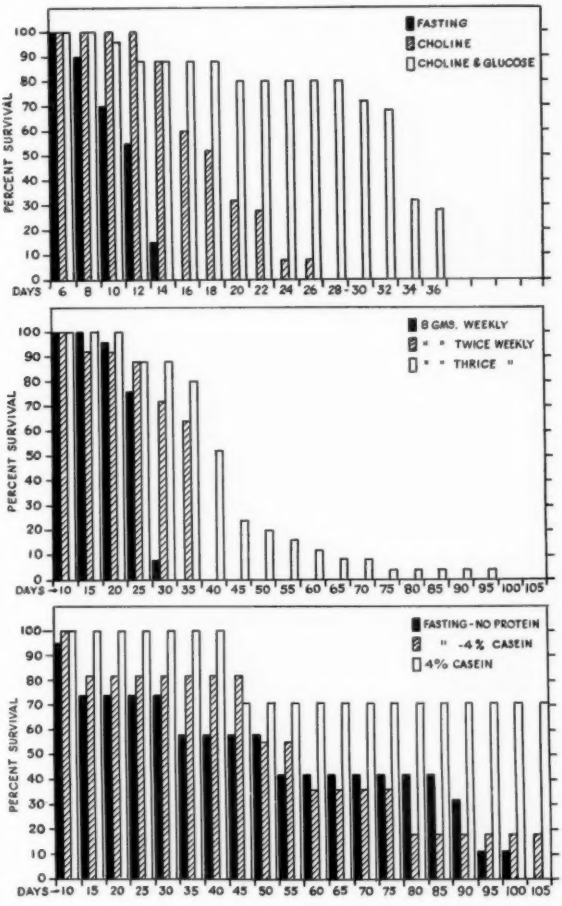

FIGURE 5.—Percent survival of rats on different dietary treatments as indicated.

TABLE 2.—*The minimum, maximum, and average survival time of rats under various dietary regimens*

Diet No.	Number of animals	Survival		
		Minimum	Maximum	Average
		Days	*Days*	*Days*
70	20	6	14	11.1
90	25	12	26	18.0
91	25	8	52	36.4
70-89	25	19	31	25.2
70-93	25	11	37	31.7
70-92	25	21	96	41.9
70-82	19	9	104	54.4
70-78	22	11	108	58.3
78	7	41	239	125.3

sis had occurred to make the diagnosis questionable.

PATHOLOGY

The stomachs were opened at the time of autopsy and examined immediately. The fixatives and stains used were Zenker fixation for the haematoxylin and eosin stain, Glynn's stain for bacteria, and Stein's stain for bilirubin; sublimate-formol [1] fixation for Mayer's mucicar-

[1] *Formalin full strength, 1 part; saturated aqueous sublimate, 3 parts.*

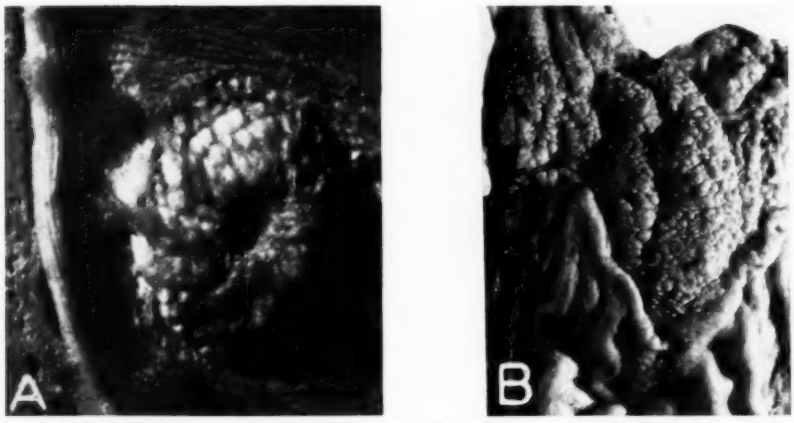

FIGURE 6.—*A*, Single papilloma of forestomach after 4 days of fasting. × 25; *B*, Multiple papilloma of forestomach after 9 days of fasting. × 4.

mine stain, and Heidenhain's aniline blue stain for mucin and parietal cells. Stomachs from 255 rats were studied microscopically.

Gross evidence of lesions in the forestomach was first observed 4 to 5 days after complete deprivation of food. At this time the entire stomach appeared smaller than normal. In the forestomach some of the mucosal folds were accentuated and packed together in parallel rows. Other portions of the mucosa remained relatively smooth, and discrete papillomas (fig. 6, *A*) were found scattered over its surface. These papillomas ranged in size up to 30 mm. in diameter. They were dome-shaped and often contained a central depression. By the tenth to fourteenth day papillomas were so numerous throughout the entire mucosa that it often appeared diffusely beaded (fig. 6, *B*). Papillomas were never found in the glandular portion of the stomach. In the latter chamber, from the fifth to the fourteenth day, superficial ulcers were observed. They varied in color and size from hemorrhagic pin-point areas to greenish-yellow circular defects 20 mm. in length.

Microscopically, the papilloma of the forestomach was recognized as having a thin core of compressed connective tissue with overlying proliferating squamous epithelium (fig. 7). Occasionally this connective tissue was edematous. Edema was also present at times beneath normal epithelium (fig. 8, *A*). The size of the papilloma varied with the extent of epithelial hyperplasia. The keratin was arranged externally in fibrillary layers as well as in concentric masses. In some instances the keratin was destroyed by ulceration which at times extended through the epithelium. Ulceration also occurred in nonpapillomatous areas (fig. 8, *B*). Beneath the keratin the squamous cells were increased in numbers. In the basal region the cell nuclei were prominent but not hyperchromatic. Mitotic figures were frequently seen with as many as 12 to the high-power field. In this portion of the papilloma many spurs of hyperplastic epithelial cells projected down to the muscularis mucosae (fig. 9, *A*). The basement membrane underlying these groups of cells remained intact. In only one instance was such a spur seen to invade the muscularis mucosae (fig. 9, *B*).

Beneath the muscularis mucosae the connective tissue was frequently edematous and contained eosinophiles and a few other polymorphonuclear leukocytes. Lymphocytes and plasma cells were rarely present. No bacteria were detected in the edematous tissue by the Glynn stain for gram-positive and gram-negative organisms. The muscular coats and serosa were normal.

It was observed microscopically that a circulatory disturbance initiated the changes resulting in the formation of the mucosal ulcers of the glandular portion of the stomach. The peripheral capillaries, in focal areas, became hyperemic, and this condition was followed by escape of red blood cells. The spread of the red blood cells was restricted at first to the relatively small amount of loose connective tissue lying between the intact acini. During this period the parietal and chief cells of the acini appeared normal and the neck cells were still filled with mucus. As stasis and hemorrhage increased, mantles of red blood cells became confluent (figs. 10 and 11). At this time many of the mucus-secreting neck cells disappeared, but some of them persisted (fig. 12, *A* and *B*). Simultaneously the acini began to lose their normal cellular arrangement. The lumens of acini disappeared as the margins of the cells became indistinct. Nuclei of adjacent cells became pyknotic. Shallow ulcers formed as the result of whole groups of acini becoming necrotic, together with the destruction of the overlying layer of mucus cells (fig. 13). In the haematoxylin and eosin sections the ulcers often appeared yellowish-green and

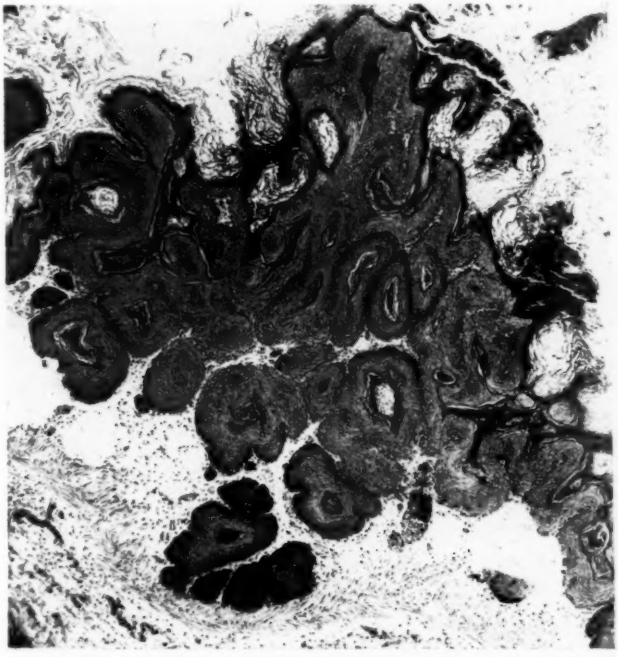

FIGURE 7.—Papilloma of forestomach in animal that fasted 7 days, and was then given three 8-gm. feedings of control diet at weekly intervals. × 55.

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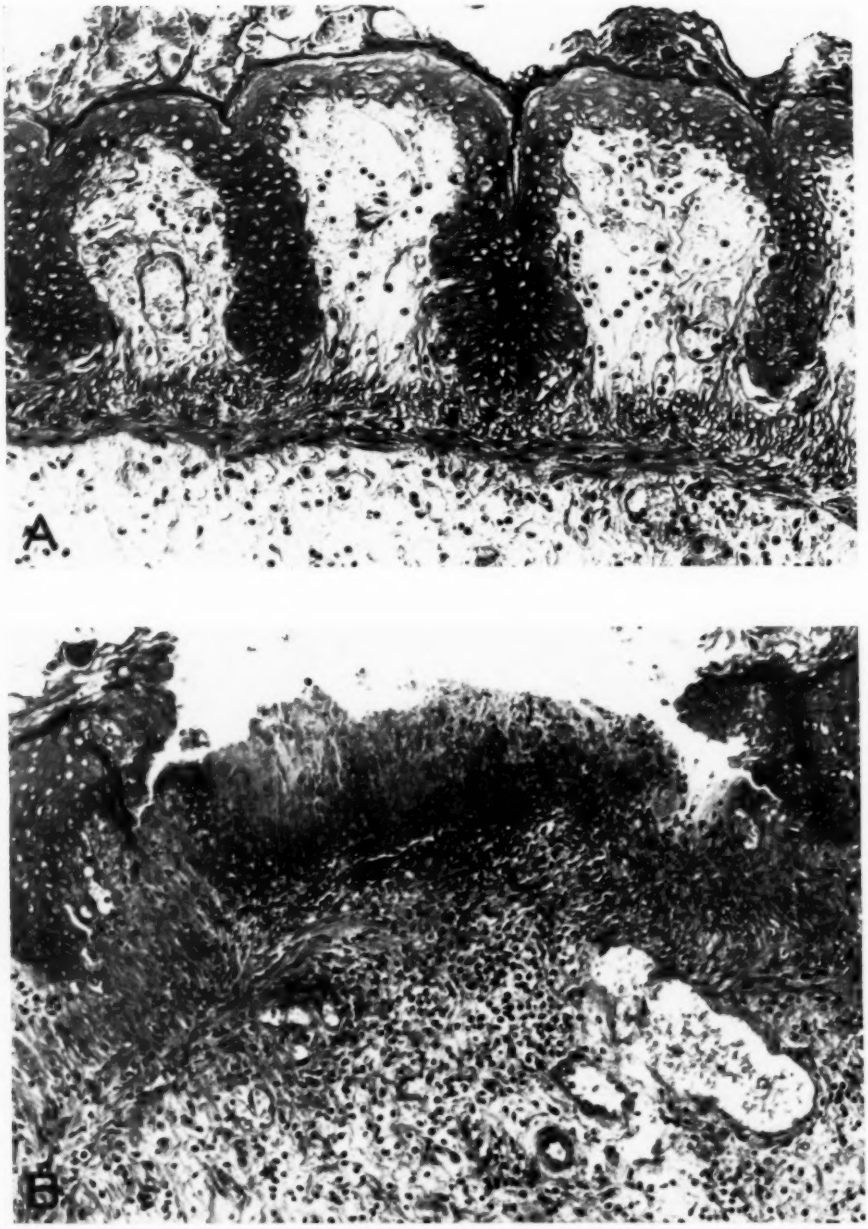

FIGURE 8.—*A*, Edema beneath squamous epithelium in animal that fasted 7 days. × 200; *B*, Ulceration of forestomach, in non-papillomatous area, of animal that fasted 7 days. × 200.

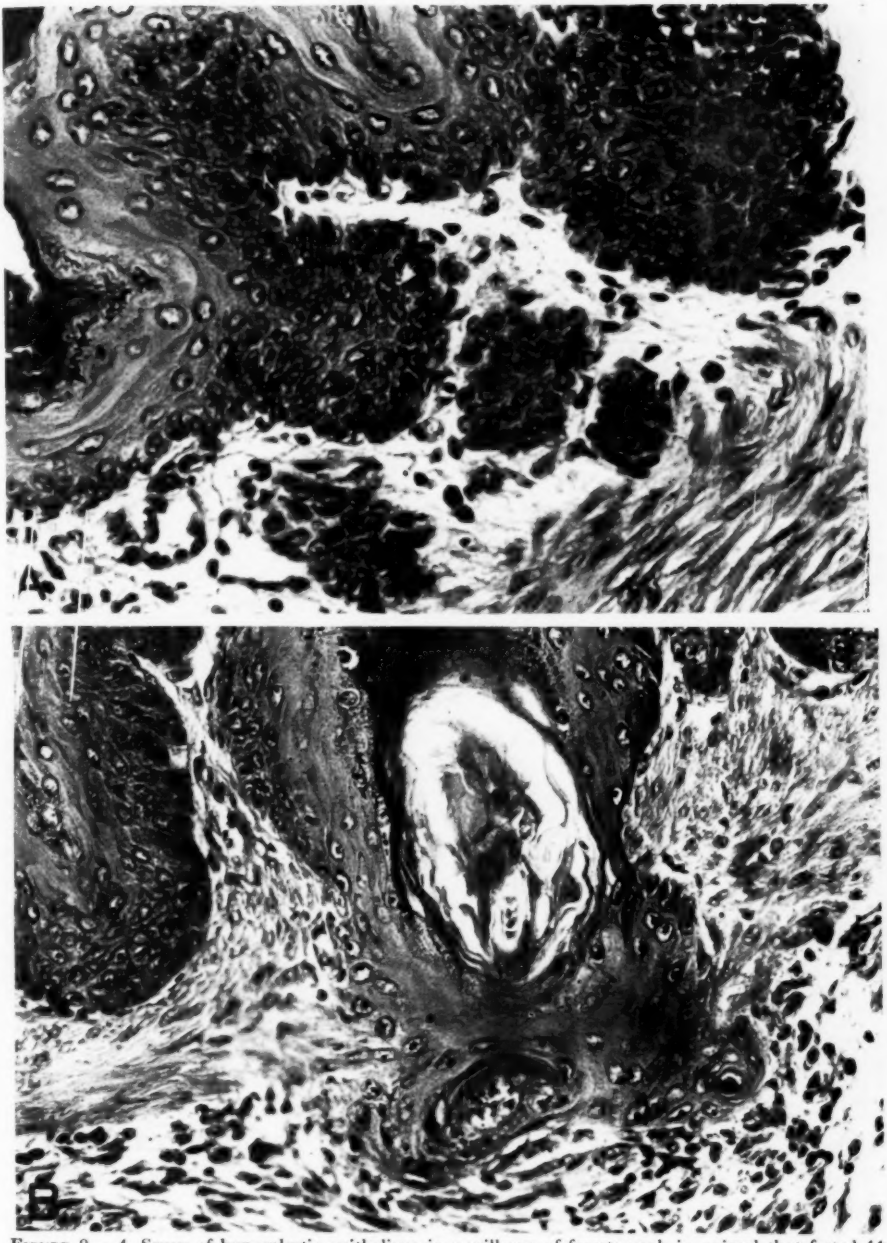

FIGURE 9.—*A*, Spurs of hyperplastic epithelium in papilloma of forestomach in animal that fasted 11 days. × 500; *B*, Spur of hyperplastic epithelium from a papilloma of the forestomach penetrating muscularis mucosae in animal that fasted 13 days. × 355.

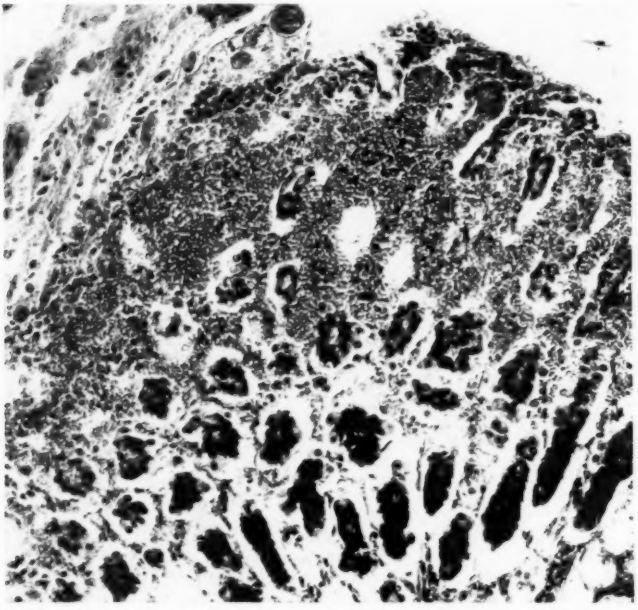

FIGURE 10.—Diapedesis of red blood cells about superficial capillaries of the gastric mucosa with beginning degeneration of acini in the early stages of formation of an ulcer in an animal that fasted 10 days. × 235.

this suggested the presence of bile deposition. However, with Stein's technique bilirubin was not found to be present. With the loss of overlying necrotic tissue the margins and bases of these ulcers became clearly defined. Inflammatory exudate was absent. The ulcers were usually limited to the outer half of the mucosa but occasionally extended down to the muscularis mucosae.

In the development of this acute type of mucosal ulcer, it is apparent that the process did not originate with a primary local loss of continuity in the layer of mucus-

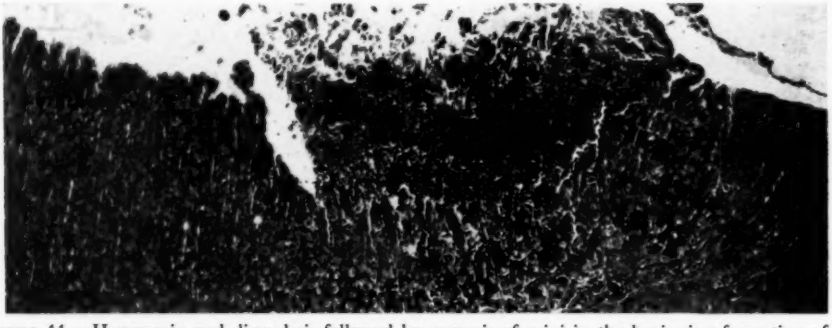

FIGURE 11.—Hyperemia and diapedesis followed by necrosis of acini in the beginning formation of an ulcer of the glandular portion of the mucosa in an animal that fasted 7 days. × 105.

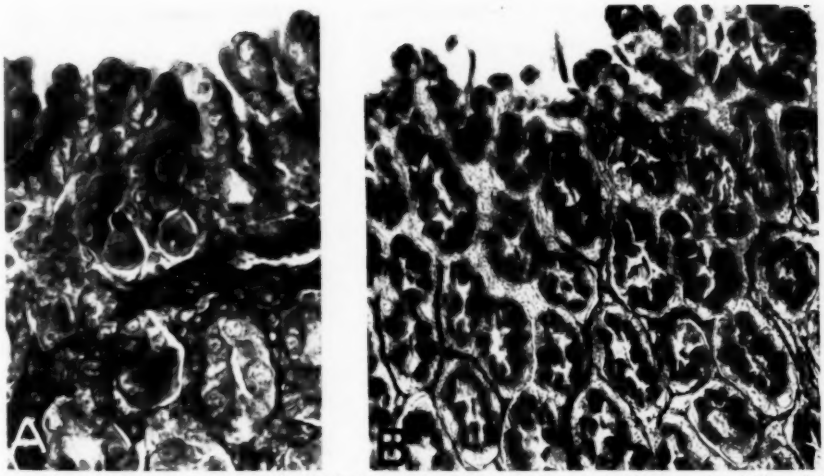

FIGURE 12.—*A*, Mucosa stained by Heidenhain's aniline blue stain for mucin showing the ability of these cells to secrete mucus after animal had fasted 11 days. × 515; *B*, Similar preparation from an animal fasting for 22 days with addition of choline only to the drinking water. Even in this state of inanition the animal is still secreting mucus abundantly. × 380.

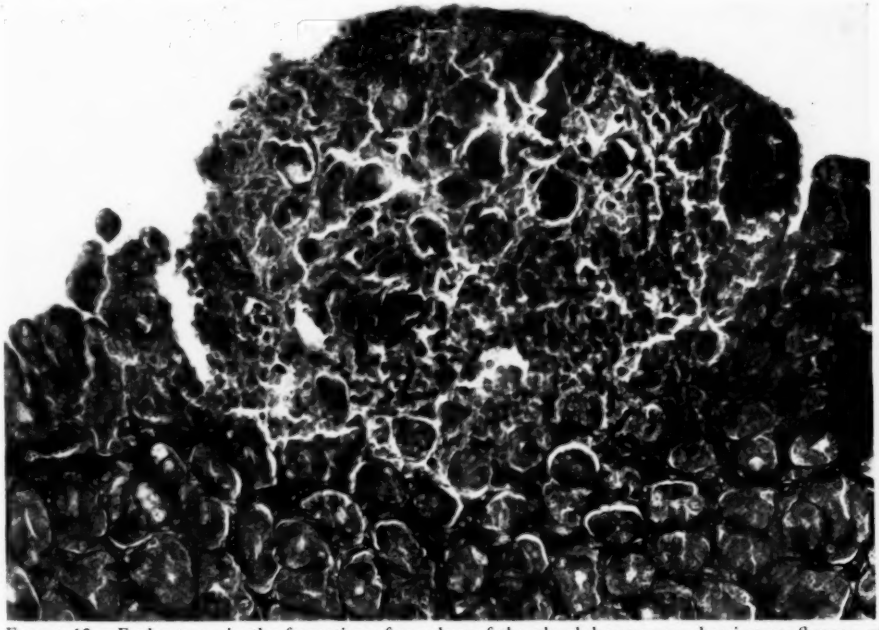

FIGURE 13.—Early stages in the formation of an ulcer of the glandular mucosa showing confluence of escaped red blood cells, acinar necrosis, and partial loss of ability to secrete mucus in overlying mucus neck cells. The marginal line of necrosis shows this tissue separating from the viable adjoining tissue. Heidenhain's aniline blue stain for mucin in an animal that fasted 11 days. × 310.

TABLE 3.—*Histopathologic analysis of stomach lesions observed following various dietary regimens*

Diet No.	Description of diet	Number of rats	Mean survival period	Stomach lesions and location (in parentheses)	
				Papilloma (forestomach)	Ulcer (glandular stomach)
	FASTING AND PARTIAL INANITION REGIMEN				
			Days	*Percent*	*Percent*
70	Fasting; water available	58	11.1	59	19
90	Fasting (choline in H_2O)	22	18.0	73	45
91	Choline in 25-percent solution glucose	24	36.4	39	0
70-89	8 gm. complete diet once weekly preceded by a 7-day fast.	21	25.2	48	48
70-93	8 gm. complete diet twice weekly preceded by a 7-day fast.	20	31.7	22	33
70-92	4 gm. complete diet 3 or 4 times weekly preceded by a 7-day fast.	15	41.9	7	27
	PROTEIN-DEFICIENT REGIMEN				
70-81	Complete synthetic diet (18-percent casein). Control preceded by a 7-day fast.	21	[1] 108	5	0
70-78	Complete low protein (4 percent casein) preceded by a 7-day fast.	18	58.3	44	0
78	Complete low protein (4 percent casein) not preceded by a 7-day fast.	4	125.3	25	0
70-82	Complete except for protein (no protein) preceded by a 7-day fast.	17	54.4	6	0
	CARBOHYDRATE-DEFICIENT REGIMEN				
70-88	Complete except for carbohydrate, preceded by 7-day fast.	8	[1] 140	13	0
88	Complete except for carbohydrate, not preceded by fast.	10	[1] 140	30	0
70-88+88		18		22	0
	FAT-DEFICIENT REGIMEN				
70-94	Complete except no fat (18 percent casein) preceded by a 7-day fast.	8	[1] 140	0	0
94	Complete except no fat (18 percent casein) not preceded by fast.	9	[1] 140	11	0
70-94+94		17		6	0

[1] End of experiment.

secreting cells. Instead, there was a multiple local circulatory disturbance and subsequent interference in the nutrition of all the cells of that region. This resulted in necrosis of acini and the formation of superficial mucosal ulcers.

The percentage of ulcers developing or remaining as a result of each dietary regimen as well as the percentage of papillomas is recorded in table 3.

This table was assembled following the microscopic examination of each animal. The histopathology was completed without knowledge of the dietary regimens in order to furnish unbiased data concerning the effects of the various dietary procedures. Following compilation of the data it was found, as shown in table 3, that fasting and partial inanition resulted in the formation of both papillomas of the forestomach and glandular ulcers, but glandular ulcers were entirely absent after an average of 36 days of feeding the aqueous solution of glucose containing choline. Papillomas were present in this group, however, to the extent of about 40 percent.

It seems evident from table 3 that the presence of glandular ulcers in these experiments is due to complete or partial inanition, but the extent of ulcer forma-

tion is related also to the length of survival as well as to the frequency of feeding. In the fasting animal surviving an average of 11 days, the percentage of ulcers and papillomas was less than in the group receiving only choline in the water, but with an average survival of 18 days. In the partially fed groups there was a decrease in the percentage of papillomas and glandular ulcers with increase in frequency of feeding. This indicates that intermittent feeding of small amounts of a complete diet has a protective action; the frequency of feeding is apparently more effective than the quantity of food eaten for the group fed 16 gm. in two weekly feedings still had more papillomas and ulcers than the group fed only 12 gm. divided into three weekly feedings.

The presence of food available ad libitum, even though it was completely devoid of protein, provided for the recovery from the glandular ulcers present after 7 days of fasting. Papillomas still remained in all groups; the percentage was very low, however, in the groups receiving the complete diet, the protein-free diet, and the fat-free diet. The 4 percent casein diet, on the other hand, did not aid greatly in recovery for there were still over 40 percent of papillomas present 58 days after the start of the experiment. That this diet not only failed to permit the recovery from these lesions following the 7 days of fasting but actually aided in their formation, is suggested by the small group fed the 4 percent casein diet without the prefasting period but where average survival was nearly twice as long.

It is difficult to understand why there was more complete restitution to a normal mucosa in the complete absence of protein (diet 70–82) than when 4 percent casein was present. In our groups, 0.1 percent methionine in the 4 percent casein diet did not protect the forestomach from formation of papillomas either following fasting or without prior fasting. No glandular ulcers, however, were observed in the protein-deficient groups.

More papillomas were present in the carbohydrate-deficient regimen (table 3), than in the fat-deficient regimen. No glandular ulcers were found in either group. Clearly the presence of over 40 percent casein in the carbohydrate-deficient groups did not prevent the formation of papillomas.

DISCUSSION

Apparently, from these data, the presence of even minimal amounts of food reduces the number of papillomas as compared with the number resulting from complete fasting. Disappearance of papillomas resulting from fasting, after the animal is placed on a diet lacking in fat, occurs more frequently than when the animal is on a diet lacking in carbohydrate and in the latter case more often than when the animal is on a diet lacking in protein.

The complete absence of any of these three major dietary components does not tend to increase the number of papillomas, and under these conditions none of the papillomas become malignant. It is the presence of even small amounts of energy-producing substances of any nature that helps to prevent the formation of papillomas. This is possibly due to the fact that in the breakdown of these foodstuffs there is enough substitution of one component for another as sources of energy.

Analysis of the data on the formation of glandular ulcers of the stomach of the rat indicates that the absence of protein, carbohydrate, or fat individually are not factors in the genesis of such ulcers. It is the absence of all three components resulting in inanition that places the animal in a state that permits the ulcers to form.

This suggests the possibility that when the animals have reached a state of shock from inanition there is a histamine-like action which is responsible for the circulatory disturbance in the peripheral portion of the mucosa, subsequent interference in nutrition, local necrosis, and ulceration. This explanation is further supported by the results of subcutaneous injections of histamine reported by Büchner and Molloy (*21*) and Bürkle-de-la-Camp (*22*) who found that large amounts of injected histamine resulted in glandular ulcers.

Choline prevents the formation of forestomach lesions on a white-flour diet according to Sharpless (*14*). If choline has such a specific effect, would it also be manifested by including choline in the drinking water of the fasting animal? The data of table 3 do not indicate such a specific effect of choline in preventing forestomach lesions during fasting. The most striking effect of choline, however, in these experiments was upon the increased survival of the animals receiving this material. The fasted animals which lived longest had lost the greater portion of their visible subcutaneous fat. It is assumed that this fat had been catabolized to furnish the animal energy.

It is generally recognized that the occurrence of ketosis in the living organism may be regarded, for practical purposes, as an index of the catabolism of fat. Raper and Smith (*23*) observed that the perfused liver containing 30 percent fat produced more ketones than the liver with 3–5 percent fat. MacKay, Carne, Wick, and Visscher (*24*) reported an increase in liver fat concentration beginning about 48 hours after start of fasting, but in their experiments neither choline, methionine, or cystine administered prior to fasting had a significant effect on the degree of fasting ketosis in the rat. Deuel, Murray, Hallman, and Tyler (*25*), on the other hand, observed a lowered liver fat in rats when large dosages of choline were administered during fasting. They also observed in male rats, whose livers contained a high percent of fat prior to fasting, a lower ketonuria on the fourth and fifth days of fasting when large amounts of choline were administered. On the basis of their experiments they concluded "that choline does not increase the rate of fat oxidation (if ketonuria during the fasting is an evidence of such oxidation.)" The fact that fasting rats lived longer when small amounts of choline were incorporated into their drinking water suggests an improvement in the functioning of the liver, which may be related to the prevention of fat deposition or utilization in the liver and possibly in prolonged fasting to the retarding effect of choline upon the formation of ketones.

Brunschwig and Rasmussen (*18*) recently reported studies on the relation of diet to benign lesions of the rat's stomach. A single dietary deficiency was not present in any of their regimens, but usually several deficiencies, e. g., a diet containing lard only or casein only, are unsuitable for the study of the dietary relation of these lesions. It is impossible to interpret their data because of the many variables introduced in the use of such deficient regimens. In order to study the causal dietary factors for such lesions, it is believed that only a single variable at a time should be studied. A beginning of such a comprehensive study is reported here. Brunschwig and Rasmussen used young and relatively small rats, and their experiments were of short duration. In their fasting experiments the survival time was only 4 days. This short survival time might be related to the temperature of the animal room (not stated), for a low animal-room temperature below the critical temperature of the rat would markedly

decrease the length of time a fasting rat would survive. In our experiments the average survival period for the fasting animals was 11.1 days, almost three times the length of survival in the fasting experiments of Brunschwig and Rasmussen.

Papillomas were not prevented, in our studies, on diet 78 containing 4 percent casein plus 0.1 percent methionine. Sharpless (*11*) found lesions of the stomach did not occur either when the level of casein was raised to 12 percent or when 0.2 percent cystine was added to the low protein diet, as Sharpless' diet also contained 5 percent of dried yeast as a source of vitamins which contributed another 2.25 percent of protein to the diet; it is still unexplained whether or not the added cystine at a lower level of total protein, such as we had, would still be effective in the prevention of the papillomas in the forestomach.

Howes and Vivier (*8*) observed, on their diet No. 84, a decrease of from 6–8 gm. of daily food consumed per rat at the beginning of the experiment to about 4–5 gm. daily. Most of the lesions in their animals were in the rumen, but a few were in the glandular portion of the stomach indicating that they were probably dealing with partial inanition as well as inadequate protein and probably vitamin deficiencies.

Hoelzel and DaCosta (*9–10*) reported that starvation was the most effective means of producing forestomach lesions in rats. They were of the opinion that diets entirely devoid of proteins were about as effective in producing the lesions as starvation. They used 5 to 6 percent of yeast in their diets as a source of the B vitamins. This yeast contributed a considerable amount of protein to their so-called protein-free diet. The actual composition of the diets was not given, and therefore it is impossible to evaluate their findings as related to the dietary factors.

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No ulcers and only a very low percentage of papillomas, 6 percent, or 1 animal in 17, were found in our experiments on a protein-free diet following a 7-day fasting period. The same percentage was found in the dietary group low in carbohydrate but containing 41.5 percent casein.

It is possible that the results which we obtained in our protein-deficient group were due to the high fat content of our protein-deficient diets and the sparing action of fat upon the thiamin requirement of the animal. Satisfactory food intake (3 or more grams daily) was obtained even on a diet entirely devoid of protein, but containing 50 percent fat during which time the animal was losing weight. If our diets had been low in fat and high in carbohydrate, the food intake would probably have been adversely affected, consequently resulting in a lower thiamin intake, when actually the thiamin requirement would be greater because of the absence of the sparing action of fat toward thiamin. Thus in reality such a regimen might actually result in a more or less chronic state of partial inanition. It is believed that further information could be obtained by additional experiments utilizing thiamin deficiency of varying degree to control food intake thus actually securing by dietary means a condition of partial inanition.

The experiments so far completed would indicate that the absence of protein, carbohydrate, or fat individually is not the specific factor in the formation of either the papilloma of the forestomach or the ulcer of the glandular stomach in the albino rat.

SUMMARY

Fourteen experimental groups of animals were studied to observe whether fasting, partial inanition, specific deficiency of protein, fat, or carbohydrate were asso-

ciated with the genesis of papillomas of the forestomach or ulcers of the glandular portion of the stomach in rats. Microscopic examination of the material was made on a total of 255 rats.

Fasting and partial inanition were found to be more important in the genesis of papillomas of the forestomach than the individual deficiency of protein, carbohydrate, or fat.

The presence of small amounts of energy-yielding food constituents was more effective than the presence of any specific dietary deficiency in preventing the formation of the papillomas of the forestomach.

The frequency of intermittent feeding was found to be more effective than the quantity of food in prolonging the life of the rat.

There was a tendency for papillomas of the forestomach to disappear after a period of less than 3 months even though the diet lacked either protein, fat, or carbohydrate, and under such dietary deficiencies there was never transformation from a benign to a malignant neoplasm.

Superficial ulcers of the glandular portion of the stomach developed during fasting and partial inanition but never were observed during the individual absence from the diet of fat, carbohydrate, or protein.

Small amounts of choline in the drinking water increased the survival of fasting rats but had no specific effect in preventing lesions of the forestomach resulting from fasting.

A regimen consisting of choline in a 25-percent aqueous glucose solution prevented the formation of glandular ulcers in an otherwise fasting rat.

A circulatory disturbance in the peripheral capillaries of the glandular mucosa with subsequent local interference in nutrition resulted in necrosis of acini and overlying mucus-secreting cells, with the formation of superficial mucosal ulcers. It was not primarily a break in continuity of the layer of mucus-secreting cells per se which initiated the formation of this type of ulcer.

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A Transplantable Malignant Hemangioendothelioma of the Liver in the Mouse

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In this paper is reported a transplantable primary malignant hemangioendothelioma of the liver arising spontaneously in a male mouse of the C3H strain. The purpose is (1) to put on record the occurrence of such a tumor in the mouse, and (2) to provide evidence indicating that in the case of hemangioendothelioma in this animal as in man, the histologic picture may not always be correlated accurately with the behavior of the tumor. This tumor is referred to in our laboratory as 98/24.

HISTORY OF TUMOR

The neoplasm arose spontaneously in the liver of a 15-month-old male C3H mouse of the Andervont line. At autopsy on September 13, 1940, there was no evidence of tumor elsewhere than in the liver. At this time a portion of the growth was fixed, and the rest was transplanted by the trocar technique to the subcutaneous tissue of four mice of the homologous strain and line.

The transplants grew successfully in each of these animals, two showing multiple hepatic metastases at autopsy. The rate of growth of the subcutaneous transplants varied so that in the four first-generation mice a tumor 1.5 cm. in diameter was present at 8, 13, 13, and 14 months, respectively. The hepatic metasteses appeared in the first two of these animals. Transplantation has been continued from one of the subcutaneous transplants and from a metastasis in the liver of this mouse. These two lines of transplantation are described jointly below.

The rate of growth has increased from the second generation so that now a tumor 1.5 cm. in diameter is present in 3 to 4 weeks after transplantation, and the incidence of successful transplants has been high, averaging about 80 percent. In the line carried from the first subcutaneous transplant, the tumor is now in the fourth generation; and in the line from the hepatic metastasis the transplant is in the fifth generation removed from the metastasis.

GROSS PATHOLOGY

Primary Tumor

The growth was a solitary reddish-purple soft mass measuring 0.8 cm. in diameter occurring in the liver which otherwise was not remarkable in appearance. The mass bulged above the surface and was covered by a thin, glistening, intact capsule.

Subcutaneous Transplants

Most of the transplants were examined pathologically after they had attained a diameter of 1.5 cm. They were discrete and surrounded by edematous purple or brown connective tissue. The tumors lying in the lateral chest wall derived their blood supply mainly from axillary vessels which were greatly dilated.

In the earlier generations the growths were purple, soft, and fluctuant, and on being cut, collapsed with the escape of a relatively large amount of fluid purplish-red blood. The tissue about this collec-

tion of blood was a relatively thin shell of tough, pearly gray material 2 mm. thick. In later generations the masses have been firmer and more solid varying from gray to purple. Cutting into these usually failed to reveal a bloody cyst. Instead the tissue was tough, glistening, gray, honeycombed with blood-filled spaces, 1–3 mm. in diameter. In tumors greater than 1.5 cm. in diameter ulceration of the overlying skin often occurred.

Metastases

Metastases were observed in only two mice each carrying first-generation transplants in the subcutaneous tissue. In each case the secondaries were multiple and restricted to the liver. They were raised, soft, cystic, purplish-red masses measuring 2–10 mm. in diameter, were replacing hepatic tissue, and were covered by an intact, raised hepatic capsule. On cutting into them fluid blood escaped. In the first case the metastases gave rise to a moderate enlargement of the liver; in the second, although the masses were multiple, they were small and were not responsible for any apparent increase in size of the organ.

MICROSCOPIC EXAMINATION [1]

The animals were killed by cervical dislocation. Sections of tumor for microscopic study were taken within a few minutes after death.

Routinely, the tissues were fixed either in Zenker's solution or Zenker-formol mixture. On occasion, formaldehyde solution (1:10 U. S. P.), 95 percent ethyl alcohol, Champy with post-osmication, and Regaud with post-chromation were used additionally. The haematoxylin and eosin stain was employed routinely, and in addition the following stains were used in certain instances on suitably fixed specimens: Mallory's aniline blue connective tissue, Mallory's phosphotungstic acid-haematoxylin, Foot's modification of Bielschowsky's stain for reticulum, the potassium ferrocyanide-hydrochloric acid test for iron, Gomori's method for alkaline phosphatase (*1*), Bensley-Cowdry acid fuchsin-methyl green stain for mitochondria, and Nassonov's modification of Kolatchew's method for demonstrating the Golgi apparatus.

Primary Tumor

The primary growth was a highly vascular tumor composed of many wide, cavernous, blood-filled sinuses as well as smaller channels including capillaries (fig. 1, *A*). The lining of all of these spaces consisted of a single layer of elongated, flattened, endothelial cells with faintly eosinophilic cytoplasm. The nuclei were elongated with a relatively thick membrane, and a moderate amount of chromatin was distributed evenly in fine granules. A relatively small basophilic nucleolus was present. Mitotic figures were not observed in the primary tumor. There was one small solid area of cells.

The large blood-filled spaces were distributed unevenly, some being separated by narrow septa, while in other situations the intervening tissue was composed of neoplastic tissue showing a delicate framework of thin collagenous fibers among which were blood-containing tortuous capillaries and endothelial-lined clefts (fig. 1, *B*). The narrow septa covered by endothelium were composed completely or partially of collagen, which in some areas contained varying numbers of elongated cells similar to the lining endothelial cells. In still other situations, the individual septa were composed essentially of a single-layered cord of well-differentiated,

[1] *In all sections from which the photomicrographs are presented in this paper except those of figure 3 the haematoxylin and eosin stain was used. In those of figure 3 Foot's modification of Bielschowsky's method for demonstrating reticulum was employed.*

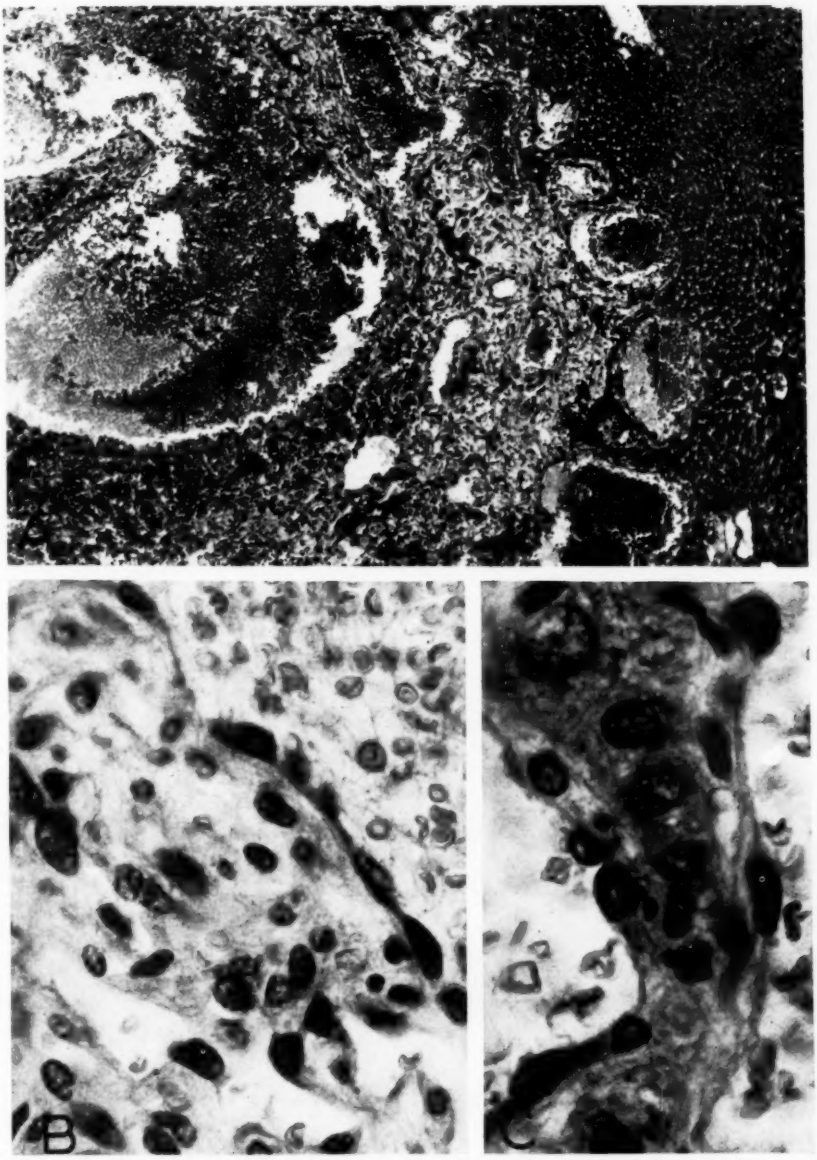

FIGURE 1.—Primary hemangioendothelioma of liver: *A*, × 80; *B*, Vascular spaces of varying size × 800; *C*, A cord of liver cells acts as a prop for tumor blood vessel walls. × 1200.

adult hepatic parenchymal cells. In the interior of the tumor such cords showed no connection with the liver. However, at the junction of tumor and liver, where there was irregular invasion of the organ, scattered thin cords of hepatic cells, connected at one end with the liver, were elsewhere surrounded by neoplastic tissue (fig. 1, *A*). Elements of hepatic tissue so caught in the tumor appeared to be acting as props for the tumor tissue in these situations (fig. 1, *C*).

Iron-containing golden-yellow pigment was present in small amount in the stroma of the tumor. The liver elsewhere showed no evident abnormality microscopically.

Subcutaneous Transplants

The transplants, growing as hemangiomatous tumors with blood-filled capillaries, cavernous spaces, and plexuses, resembled the primary neoplasm in essentials. There were some interesting and important differences, namely, complete absence of hepatic parenchymal cells, evidence of less differentiation and greater rate of growth of tumor cells, and the presence of numerous solid sheets of cells (fig. 2, *A*). The cells lining the blood-filled spaces at times lacked the flat character seen in the primary growth and assumed a cuboidal shape. This characteristic was likewise seen in the nuclei which projected as a prominent row of beads into the lumen of the vessel. In general, the nuclei were larger than those in the original growth, and mitotic figures were rather frequent. There were foci of stratification of lining cells which protruded into the lumen in a papillarylike manner. Such papillae lacked a true connective tissue stalk, but there were blood-filled clefts among these cells. The cells throughout the transplants possessed an eccentric Golgi apparatus small in size, in intimate contact with the nucleus. A small percentage of the cells showed hypertrophy of the Golgi apparatus, into a less heavy osmophilic, more open form of network. All of the cells possessed fine, twisting, filamentous mitochondria.

In some of the larger vessels lined by simple flat endothelium were concentrations of cuboidal cells lying directly beneath the surface layer (fig. 2, *B*). The endothelial-covered collagenous septa taking part in the formation of blood plexuses contained varying numbers of endothelial-like cells which were occasionally associated with red blood cells apparently lying free in the tissues (fig. 2, *C*). Particularly in the later generations, there were unevenly distributed solid sheets of spindle or stubby cells showing faintly eosinophilic cytoplasm and relatively large elongated nuclei similar to those of the endothelial cells elsewhere in the tumor (fig. 4, *A*). With Mallory's phosphotungstic acid-haematoxylin stain, no fibroglia or myoglia fibrils were associated with these cells although there were occasionally observed short, thin, faintly purple fibrils. The reticulum was characteristically seen concentrated around the periphery of the individual sheets of cells (fig. 3, *A*), while in the interior of the sheet the reticulum was scanty and showed no intimate or constant relation to the tumor cells (fig. 3, *B*). That these solid areas were not foreign to the tumor is evidenced by the fact that there were transitional stages by way of blood-filled clefts among the tumor cells to actual sinusoidal formation (figs. 4, *A*, *B*, and *C*). Further, the Golgi apparatus of the cells in both solid and vascular areas was identical, showing hypertrophy in both areas in later generations. Fine filamentous forms made up the majority of the mitochondria. In the vascular areas, the reticulum was distributed about the vessels as a supporting basement membrane (fig. 3, *C*).

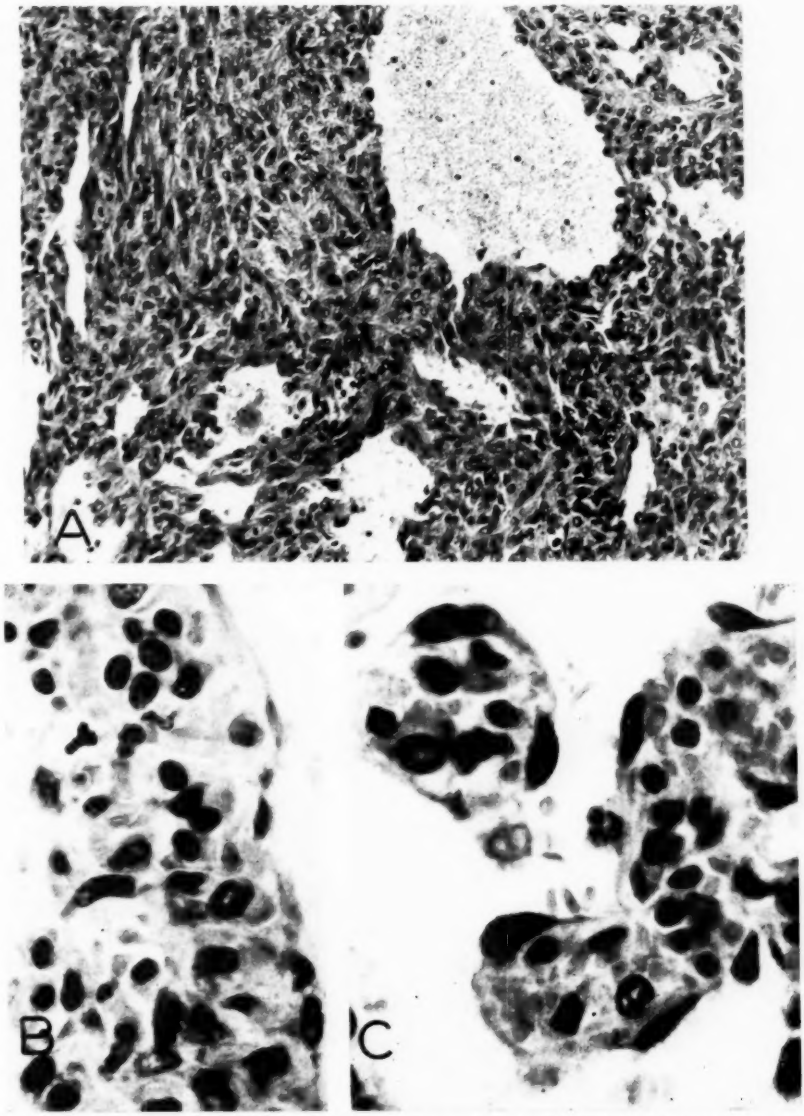

FIGURE 2.—Subcutaneous transplants: *A*, Vascular spaces of varying sizes, clefts, and solid areas. × 187, *B*, Wide vascular space lined by flat endothelium beneath which is a stratification of tumor cells. × 800; *C*, Blood plexus. Red blood cells in spaces and septa. × 1040.

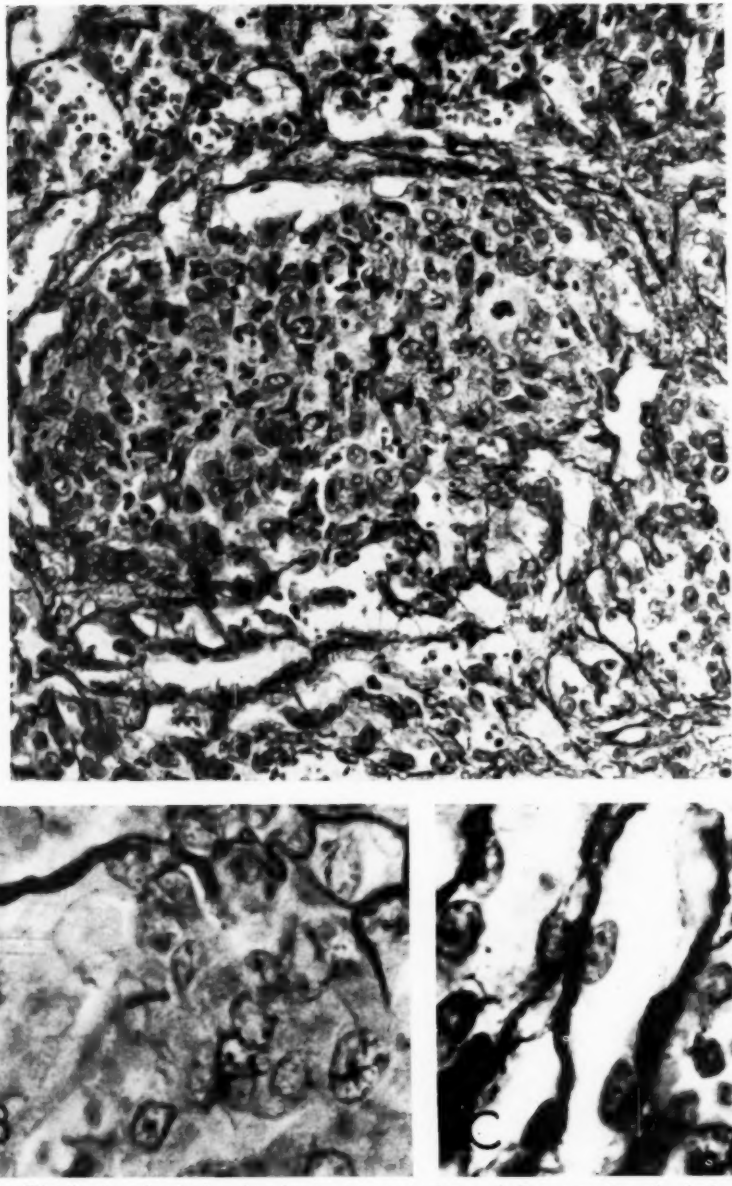

FIGURE 3.—Distribution of reticulum in subcutaneous transplants: *A*, Concentration of reticulum about periphery of solid area. × 380; *B*, Scanty and haphazard distribution in the interior of a solid area. × 1050; *C*, Basement membranelike distribution about a capillary. × 1220.

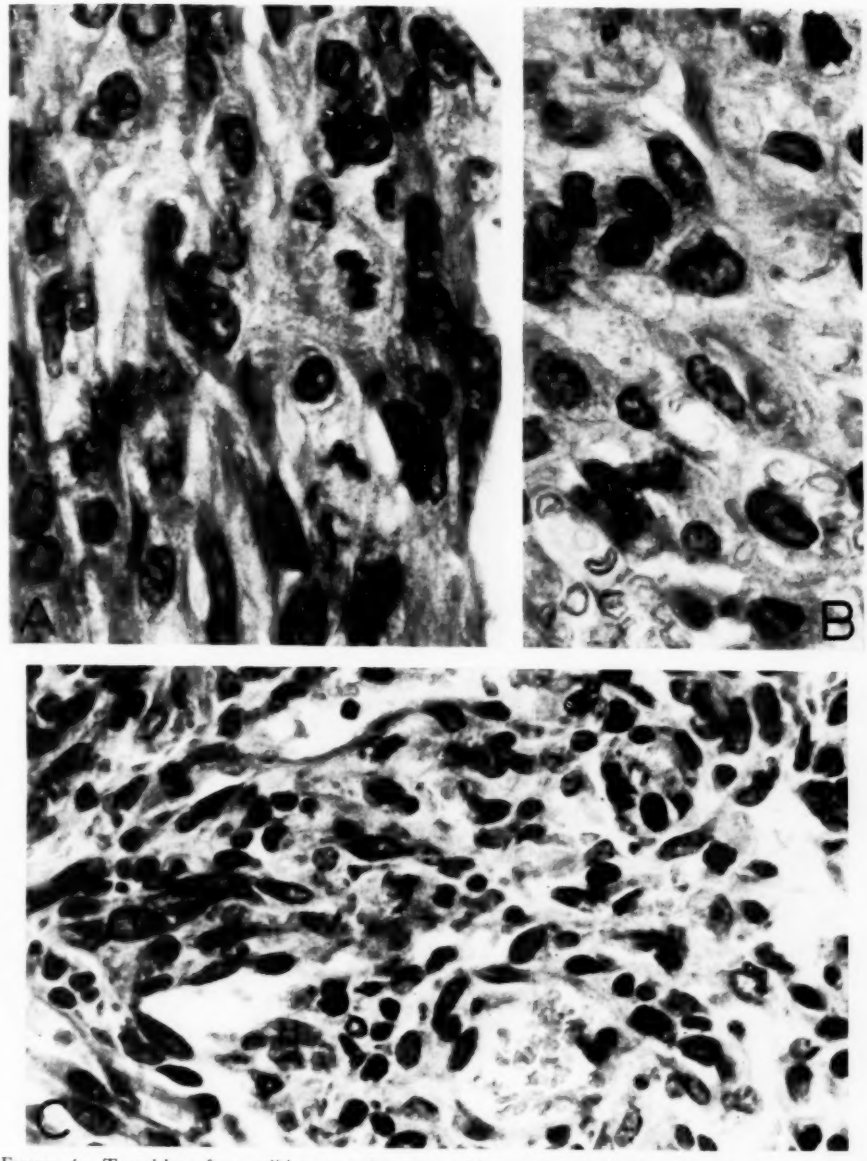

FIGURE 4.—Transitions from solid to vascular types of hemangioendothelioma in subcutaneous transplants: *A*, solid area. ×1050; *B*, Blood-filled clefts among tumor cells. × 1220; *C*, Actual vessel formation. × 400.

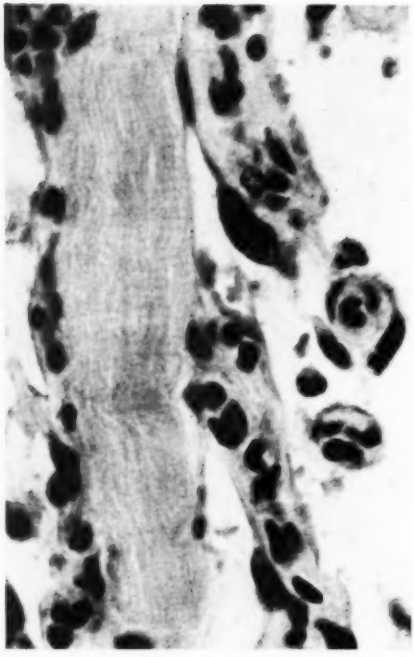

FIGURE 5.—Subcutaneous transplant. An undestroyed fiber of striated muscle acts as a prop for tumor tissue. × 800. Compare with cord of liver cells in figure 1, *C*.

The microchemical test for alkaline phosphatase of Gomori was performed on a number of representative sections of transplants and in none was any of this substance demonstrated in the tumor cells. In order to test the phagocytic powers of the tumor cells the following experiment was carried out: An aqueous suspension of india ink was injected into a soft vascular portion of a subcutaneous transplant in a living mouse. Seventy-two hours later the animal was killed and an autopsy performed. Microscopic examination revealed numerous particles of india ink in the Kupffer cells and phagocytes of the spleen, but none were found in the tumor cells. The tumors were invasive, interrupting the continuity of muscle bundles but, as in the liver, the tumor did not completely destroy tissue which it had invaded. Thus, one could observe small groups of individual fibers of muscle within the tumor. Not infrequently these appeared to serve as a support to which elements of the tumor were attached (fig. 5). The connective tissue about the tumor was edematous and showed scattered macrophages laden with iron-containing golden-yellow pigment.

METASTASES

As indicated above, metastases were found in the livers of two animals carrying first-generation transplants. The general structure of these neoplasms was very similar to that of the primary neoplasm with cords of liver cells lying in the septa between some of the blood vessels (fig. 6). Mitotic figures were present in small numbers. Although the tumor was essentially vascular, there were a few small solid areas of cells.

DISCUSSION

The question of the nature of the tumor cell and of the similarity of this nepolasm to hemangioendothelioma in man and in the mouse will be considered.

That the tumor is derived from the endothelial cell is supported by its structure in the primary site, in metastases, and in transplants. In the primary tumor the essential picture was that of completely or partially blood filled spaces lined by endothelium with adult parenchymal cells of the liver lying in some of the septa between these spaces. Casual examination might have given the impression that this tumor was a hepatoma with particularly wide stromal vessels. Contrary to such an assumption, however, was the fact that in many areas between these liver-cell cords the tumor had a purely angiomatous character as seen in figure 1. Furthermore, when the neoplasm was trans-

planted into subcutaneous tissues no hepatic parenchymal cells were to be found, but only an endothelial structure. When the tumor had metastasized from subcutaneous transplants to the liver, the presence of hepatic cell cords in some of the intervascular septa was again apparent, as in the primary tumor. It seems reasonable to consider such cords of liver cells merely as inclusions of pre-existing elements in the growth. In this way they are entirely comparable with the occasional intact fibers of striated muscle cells seen in the interior of the subcutaneous transplants.

While it was readily apparent that the vascular portions of the tumor were of a hemangioendotheliomatous nature, classification of the solid areas required more detailed analysis. Two questions naturally arose concerning the nature of such foci:

(1) Did they represent an additional new type of neoplasm? or (2) did they represent variants of hemangioendothelioma?

When primary adenomatous tumors of the lung in the mouse are transplanted to the subcutaneous tissue, the transplants may frequently take on the character of fibrosarcoma as demonstrated by Stewart, Grady, and Andervont.[2] Their observation, taken together with the occurrence of solid areas of elongated cells in our transplants, brought up the question of the presence of fibrosarcoma. A neoplasm of this type may, however, be ruled out since the cells of the tumor here described lacked fibroglia fibrils, and the mitochondria and Golgi apparatus were not characteristic of those of fibroblasts. Moreover, the distribution of the reticulum varied from that seen in fibroblastic tumors in which the cells and the reticulum fibers are closely associated, each cell being essentially encircled by reticulum fibrils. Within the solid areas of our tumor, the reticulum was scanty and distributed haphazardly while it was regularly concentrated at the outer margin of a solid sheet of cells.

[2] *These investigators are now preparing a paper on this subject.*

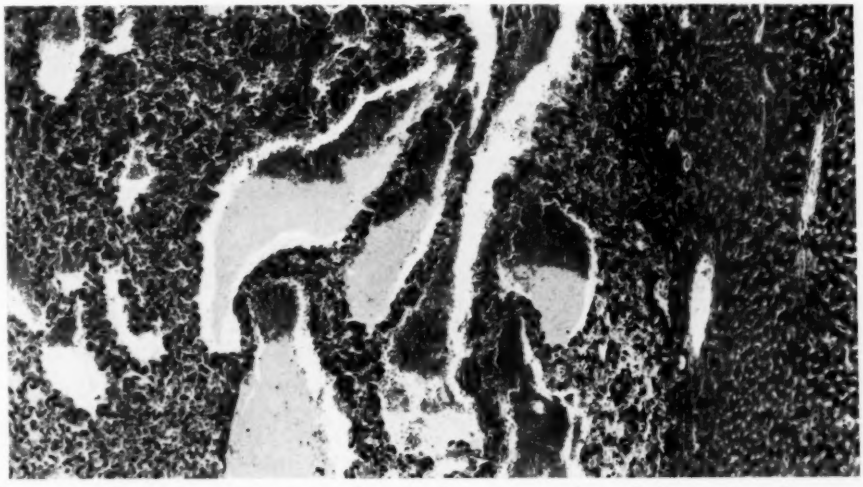

FIGURE 6.—One of many metastases in the liver of a mouse carrying a first-generation subcutaneous transplant. × 70.

Moreover, that these areas did not represent leiomyoblastoma is supported by the lack of myoglial fibrils and distribution of the reticulum which in smooth muscle tumors is similar to that seen in fibroblastomas.

In the differential diagnosis of neoplastic tissue composed of elongated cells arranged in sheets which in turn are surrounded by reticulum, one must consider the so-called spindle-cell carcinoma. In the present tumor, carcinoma was ruled out by the same factors which established the diagnosis of hemangioendothelioma, namely, mitochondria and Golgi apparatus characteristic of endothelial cells, blood-filled clefts among some of the cells in the solid sheets, and actual capillary formation in such areas.

Whether the tumor cells are specialized and derived from the Kupffer cell or from nonspecific sinus endothelium of the liver is impossible to say at this point. The lack of phagocytosis of india ink would tend to refute the Kupffer cell's being the mother cell, but this point should not be regarded as proven since a neoplastic cell does not necessarily retain the function of the normal cell from which it is derived.

In considering this tumor as derived from blood vessel endothelium, the question of malignancy naturally arises.

The primary tumor was a well-differentiated angioma of the cavernous and capillary types. The average nuclei of the cells lining the blood spaces were perhaps somewhat larger than those in normal blood vessels, but aside from this there was no remarkable difference between the cells of the tumor and the endothelium elsewhere in the body. The rate of growth, as judged by the lack of evident mitosis, was slow. It is true that the primary tumor was not encapsulated and was apparently invading adjacent hepatic tissue; but these features alone do not prove the tumor to be malignant, since it is accepted by pathologists that benign angiomatous tumors may present histologic evidence of invasion and lack of encapsulation despite their having shown no evidence of growth clinically. The general histologic picture in the case of the primary tumor suggested a benign neoplasm of blood-vessel endothelium. However, this interpretation, apparently valid on histologic grounds, is incorrect, as is proven by the fact that in two instances first-generation transplants produced multiple hepatic metastases. That these were indeed metastases rather than primary hepatic tumors is supported by their large number and wide distribution in the liver. These metastases had an essentially similar histologic picture to the primary growth. Since the ability of a tumor to metastasize is an absolute indication of malignancy, we must consider that the tumor in question was malignant regardless of its histologic appearance.

Certain of the hemangioendotheliomas in man may behave similarly to the neoplasm under discussion. Robinson and Castleman (*2*) reviewed four reported cases, those of Borrmann (*3*), Ewing (*4*), Shennan (*5*), and Geschickter and Keasbey (*6*), in each of which a histologically benign hemangioma gave rise to metastases which likewise appeared benign on microscopic examination. In their own case a histologically benign primary tumor produced metastases of an entirely different nature, lacking cystic angiomatous spaces and showing "angioblastic" small spindle cells containing hyperchromatic nuclei. They felt that their case with histologically sarcomatous metastases represented a missing link between two types of malignant hemangioendothelioma. In the first type both the primary tumor and the metastases it yielded appeared benign his-

tologically. In the second group both primary tumor and metastases appeared malignant histologically, as reported by Wright (*7*), Hall (*8*), Wollstein (*9*), and others. Since the time of Robinson and Castleman's report two other cases have been reported which should be included in their "benign metastasizing" group, namely, those of Ward and Jonas (*10*) and Parsons and Ebbs (*11*). A hemangioendothelioma histologically suggestive of malignancy with solid areas and active mitosis may respond very satisfactorily to therapy, as exemplified by the four cases of Edwards and Taylor (*12*) with "vascular endothelioma of the lung" one of which was alive 8.5 years after lobectomy. Such cases illustrate the recognized difficulty in correlating accurately the prognosis of the hemangiomatous tumors with their microscopic picture.

In 1930 Foulds (*13*) reviewed the literature on malignant tumors arising from the endothelium of the blood vessels in the mouse and also reported 9 such tumors which he personally observed. Of the cases he reviewed, 1 reported by Murray (*14*) arose in the groin of a male mouse; 3 reported by Slye, Holmes, and Wells (*15*) were primary in the scrotum, omentum, and mammary gland, respectively; and of the 3 reported by Dobrovolskaïa-Zavadskaïa and Kabozieff, 2 originated in the subcutaneous tissues and 1 in the abdominal cavity. Of Foulds' 9 cases, 1 arose in the lip and 8 in the subcutaneous tissues. In 4 of his animals metastases had occurred, in 1 to the liver, in 1 to the spleen, and in 2 to the liver and spleen. Autotransplantation was successful in 3 of 4 animals, but attempts to transplant tumors into normal mice were not successful. Similar negative results were obtained by Murray, who inoculated the tumor which he reported, into 82 mice. It is interesting that in Foulds' animals with metastases in the spleen and liver, surviving cells of the respective organs were present in the midst of the infiltrating tumor tissue, a picture seen in our primary tumor and hepatic metastases.

We have been unable to find in the literature any detailed description of primary hemangioendothelioma of the liver in the mouse although Cloudman (*16, p. 220*) states that in this organ hemangioma is common and hemangioendothelioma occurs occasionally. That hemangioendotheliomas occur commonly in the liver of man is well-recognized. Usually they are benign. In the review of 570 benign angiomas in man presented by Geschickter and Keasbey (*6*), the vast majority occurred in the subcutaneous and submucous tissues. Aside from these areas the liver represented the most common single site. There were 109 instances of primary angioma of the liver which represented one-sixth of all the benign angiomas. On the other hand, primary malignant hemangiomas of the liver in man are uncommon and usually are seen in infants (*6*).

SUMMARY

A solitary, spontaneous, transplantable hemangioendothelioma of the liver in a male C3H mouse of the Andervont line is reported.

The histologic picture of the primary tumor suggested a benign character. Its actual malignancy, however, is supported by the fact that in two animals carrying first-generation transplants metastases occurred to their respective livers. These metastases showed an essentially similar picture to the primary tumor. The transplants, in addition to showing vascular formations, as in the primary growth, presented solid areas of endothelial cells.

The difficulty of correlating the expected behavior of a hemangioendothelioma with its histologic picture is stressed.

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Disappearance of Intravenously Injected Methylcholanthrene in Mice of Different Susceptibility to Pulmonary Tumors

By Egon Lorenz, *senior biophysicist*, and Michael B. Shimkin, *assistant surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

The response of different animal species to carcinogenesis after the injection of carcinogenic hydrocarbons is widely variable. While 1,2,5,6-dibenzanthracene in doses below 0.01 mg. evokes sarcomas in mice (*1*), rabbits are relatively resistant to carcinogenesis with hydrocarbons (*2*, *3*). Dobriner, Rhoads, and Lavin (*4*), in an attempt to explain this difference in response, studied the excretion of subcutaneously injected 1,2,5,6-dibenzanthracene in mice, rats, and rabbits. They found substances of a phenolic nature, considered to be conversion products of the hydrocarbon, in the excreta of these animals. The substance isolated from rabbits was noncarcinogenic and gave absorption bands different from those of the substance obtained from rats and mice. They concluded that "this observation suggests that in certain animals an immunity to carcinogenesis by chemicals depends upon the ability to convert the carcinogen from a neutral to a phenolic compound."

Since the metabolism and elimination of carcinogenic hydrocarbons are still unelucidated, it is impossible to estimate the importance of the other influencing factors, such as diet and intestinal flora, in experiments with different species of animals.

Inbred strains of mice, which vary markedly in their response to the hydrocarbons, offer a more exact basis for this type of investigation. Thus, mice of strain A are extremely susceptible to the induction of primary pulmonary tumors with carcinogenic hydrocarbons, while strain C57 Black mice are resistant to the induction of this neoplasm. With the intravenous introduction of 0.5 mg. of 20-methylcholanthrene dispersed in horse serum, 100 percent of strain A mice develop multiple pulmonary tumors within 6 weeks; with the same procedure, only a few pulmonary tumors appear in strain C57 Black mice in 6 months (*5*).

If the resistance of mice to the induction of pulmonary tumors with methylcholanthrene depends upon the ability to conjugate and to eliminate the chemical, strain C57 Black mice should excrete or chemically alter methylcholanthrene more rapidly than mice of strain A. Conversely, the unaltered hydrocarbon should be detectable for a longer period and in higher concentration in strain A mice than in mice of strain C57 Black.

This communication reports determinations of the content of ether-extractable methylcholanthrene in the lungs and the whole bodies of strains A and C57 Black mice at various intervals after the intravenous injection of 0.5 mg. of the compound.

EXPERIMENTAL PROCEDURE

The carcinogen employed was 20-methylcholanthrene, a synthetic product with a melting point of 179.8°–180.4° C. (corrected), dispersed in horse serum saturated with cholesterol to equal 1.0 mg. of the hydrocarbon per 1.0 cc. of the vehicle. The method of preparation of the dispersion was described previously (*6*, *7*).

Mice of strains A and C57 Black were obtained from the Roscoe B. Jackson Memorial Laboratory, of Bar Harbor, Maine. The animals were kept under similar environmental and dietetic conditions (*8*). Male mice 3 months old and 25 to 30 gm. in weight were used.

The animals received a single intravenous injection, into a lateral tail vein, of 0.5 mg. of methylocholanthrene dispersed in 0.5 cc. of cholesterol-saturated horse serum. At various periods after the injection they were killed by cervical dislocation and the lungs or the whole bodies extracted for determinations of the hydrocarbon content.

Absorption Spectrum Analysis

A photographic method of absorption spectrum analysis in the ultraviolet for the detection of 1,2,5,6-dibenzanthracene in tissues was described by Lorenz and Shear in 1936 (*9*). This method is also applicable to methylcholanthrene. Figure 1 [1] shows the curve of the logarithm of the relative extinction coefficient of methylcholanthrene in dependence upon the wavelength.

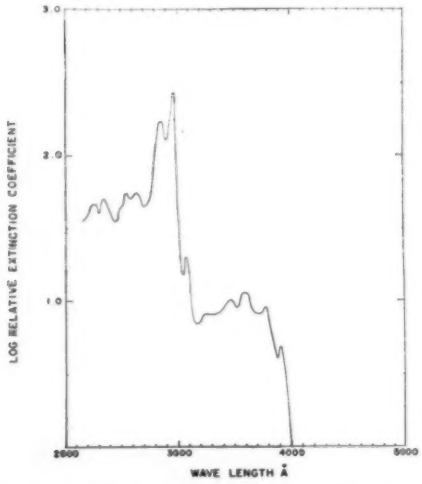

Figure 1.—Absorption spectrum of 20-methylcholanthrene dissolved in ethyl alcohol.

[1] *We are indebted to Drs. Hollaender, Cole, and Brackett* (10) *for permission to use this figure.*

For these analyses use was made of the bands lying between 4000A and 2700A, consisting of a group of bands between 4000A and 3200A and another group between 3200A and 2700A. To obtain quantitative data as well as data on possible changes in the methylcholanthrene spectrum, the spectra of the tissue extracts were compared with a set of standard spectra. More accurate data were obtained by printing on the same plate the spectra of the unknown extract and comparison spectra of standard ether solutions of the hydrocarbon in the range of concentration present in the extract. For concentrations above 0.01 mg. per cubic centimeter, the bands between 4000A and 3200A were used; for lower concentrations these bands disappear, but the bands lying between 3200A and 2700A become evident. The structure of this group of bands is not sufficiently detailed for the ready identification of methylcholanthrene in an unknown solution. However, this group of bands can be used for identification if methylcholanthrene is the material used in the experiment. It is probable that chemical changes of the hydrocarbon would manifest themselves in a shift and change of the band spectrum. Different materials present in the extracts, such as lipoids, do not change the spectrum since methylcholanthrene is a nonpolar substance. Tests in which known amounts of methylcholanthrene were added to ether extracts of tissues showed that the amount of methylcholanthrene added could be determined quantitatively within the limits of error given below. The smallest amount of methylcholanthrene detectable by the method is approximately 5×10^{-4} mg. per cubic centimeter for a 1-cm. cell.

The smallest amount of methylcholanthrene detectable in tissue depends upon the volume of the extract. Usually volumes of extracts ranged from 2 to 50 cc.

The error of this method depends upon a number of factors. The main sources of error are biological variations and the error produced in matching standard and unknown spectra. To obtain a measure of the magnitude of these factors, 12 mice of different sex, age, and strains were injected intravenously with 0.5 cc. of a methylcholanthrene-horse serum dispersion containing 1 mg. of the hydrocarbon per cubic centimeter. The animals were killed 5 minutes after injection, the lungs extracted by the method given below, and the extracts subjected to absorption spectrum analysis. The coefficient of variation of individual estimates was found to be ± 9.7 percent. The limits of variation corresponding to $P=0.05$ were estimated to be ± 21 percent. At later periods after injection, when the concentration of the hydrocarbon decreases progressively, the matching of the unknown with the known spectra becomes increasingly more difficult as differences in density between bands and the adjacent continuous spectrum become smaller. It is estimated that the total limits of variation near the limit of sensitivity of the method is of the order of magnitude of $\geqq \pm 50$ percent.

Preparation of Extracts

The lungs of the injected animals were minced with a pair of scissors. The whole bodies were ground in a meat grinder. The wet tissues were placed in 250-cc. glass-stoppered pyrex bottles. About 50 cc. of ethyl ether was added to the lungs and about 150 cc. to the whole mouse tissues. The bottles were shaken vigorously in a shaking machine for 2 to 3 hours. The ether was decanted, replaced by fresh ether, and the procedure repeated. The residue was washed three times with a total of about 75 cc. of ether.

The ether was evaporated down to 5 or 10 cc. and the extract subjected to absorption spectrum analysis. For the pulmonary tissues the ether extraction produced solutions that were sufficiently free from substances interfering with the determinations. With other tissues, and especially when the methylcholanthrene content was low, the continuous absorption produced by extracted lipoids and pigments blotted out the characteristic spectrum bands on the photographic plate. In some cases, where the extracts contained more than 0.1 mg. of methylcholanthrene, dilution of the extract was sufficient to show the absorption spectrum of the hydrocarbon.

Whole mouse extracts, varying from light yellow to deep-brown in color, had to be extracted further. The method of "partition between different solvents" (*9*) was used. The ether extracts were dried at 40° C. The residue was dissolved in 10 cc. of hexane (spectroscopically free from benzene), 2 cc. of 40 percent ethyl or methyl alcohol was added, and the mixture shaken vigorously for a few minutes. Upon standing, the liquid separated into two layers, the lower one (alcoholic) being quite turbid and containing some of the interfering substances. Absorption spectroanalysis showed that the methylcholanthrene remained in the hexane layer.

The continuous absorption was usually most prominent in the darker colored extracts. It was suspected that the color was due to oxidation of some of the interfering substances. Extraction under nitrogen was therefore undertaken and gave uncolored or lightly colored solutions

with a greatly reduced content of interfering substances.

The extraction of wet tissues with ethyl ether at room tempersture was chosen in preference to Sohxlet extraction of dried tissues because extraction of dried material results in a considerable increase in the amount of interfering substances having continuous absorption in the ultraviolet, and because the procedure is simpler and avoids any possibility of chemical change of the hydrocarbon in the tissue due to heating.[2]

The reliability of the wet-tissue extraction was shown by the recovery of the total 0.5 mg. of methylcholanthrene from the body 5 minutes after injection. Another control of the method was obtained by drying *in vacuo* over anhydrous phosphoric pentoxide 10 pooled pairs of lungs from mice that were injected with 0.5 mg. of methylcholanthrene 3 days previously. The dried tissue was extracted with ethyl ether for 24 hours in a Sohxlet apparatus. Absorption spectrum analysis revealed that the content of the hydrocarbon was 3×10^{-1} mg., or 3×10^{-2} mg. per mouse. This value checked with the results obtained with the cold ether extraction of wet lung tissue. Further evidence that the method was quantitative and that no spectroscopically detectable derivatives of the hydrocarbon remained in the tissues was obtained with extracts of tissues 2 days after the injection of methylcholanthrene. The tissues were extracted 3 times by shaking with ether and only the first 2 extracts were pooled. Absorption spectrum analysis showed that no methylcholanthrene was present in the third extraction. The tissues were then acidified to pH 3.5 with HCl or H_2SO_4, and extracted again with ether by shaking. No methylcholanthrene or derivatives of methylcholanthrene were detected in the extracts.

In order to verify that the disappearance of methylcholanthrene was due to its elimination or metabolism by the body and not to changes resultant from the contact of the hydrocarbon with organic tissues *in vitro*, the following experiment was performed: Two mice were injected each with 1.0 mg. of methylcholanthrene. They were killed 5 minutes later, and the ground carcasses were permitted to autolyze for 4 days. The content of methylcholanthrene extracted was the same as that in mice injected with the same quantity of methylcholanthrene but extracted immediately.

Experiment 1

Ten strain A and ten strain C57 Black mice were injected intravenously with 0.5 mg. of methylcholanthrene dispersion. Two animals of each strain were killed at the following intervals after injection: 30 minutes, 1 day, 3, 6, and 9 days. The lungs were removed from the body, and the methylcholanthrene content was determined. The results are presented in table 1, in which the values are given for single mice.

Experiment 2

The foregoing experiment was repeated, one mouse of strain A and one of strain C57 Black being killed at 5 minutes, 1 day, 2, 4, and 8 days after injection. The results are also given in table 1.

[2] *Fieser and Hershberg* (11) *found that methylcholanthrene can be oxidized at room temperature with lead tetraacetate. The reactivity of the hydrocarbon is illustrated by a recent experience during our experiments, in which a dried ether extract of liver tissue containing methylcholanthrene was dissolved in methyl alcohol and accidentally boiled for several hours. The extract showed a band spectrum different from methylcholanthrene, indicating that a derivative of the compound was formed by the procedure. This new band spectrum was not present in similar unheated extracts.*

TABLE 1.—*Disappearance of intravenously injected methylcholanthrene (0.5 mg.) from lungs of mice of strains A and C57 Black*

Experiment No.	Time after injection	Methylcholanthrene content per mouse	
		A mice	C57 Black mice
		Milligrams	*Milligrams*
1	30 minutes	2.5×10^{-1}	2.5×10^{-1}
	1 day	1.3×10^{-1}	$.9\times10^{-1}$
	3 days	3.2×10^{-2}	2.5×10^{-2}
	6 days	$\sim3.1\times10^{-3}$	$\sim4.6\times10^{-3}$
	9 days	$\sim1.5\times10^{-3}$	$\sim1.0\times10^{-3}$
2	5 minutes	3.0×10^{-1}	3.0×10^{-1}
	1 day	1.5×10^{-1}	1.8×10^{-1}
	2 days	7.6×10^{-2}	9.0×10^{-2}
	4 days	3.5×10^{-2}	2.8×10^{-2}
	8 days	$\sim4.0\times10^{-3}$	$\sim2.0\times10^{-3}$

EXPERIMENT 3

Seven mice of strain A and seven mice of strain C57 Black were injected with 0.5 mg. of methylcholanthrene. Two animals of each strain were killed and the whole bodies extracted 5 minutes, and 1 day after injection. One mouse of each strain was extracted 2, 3, and 4 days after the injection. The results of the absorption spectrum analyses are presented in table 2.

TABLE 2.—*Disappearance of intravenously injected methylcholanthrene (0.5 mg.) from whole body of mice of strains A and C57 Black (experiment 3)*

Time after injection	Methylcholanthrene content per mouse	
	A mice	C57 Black mice
Minutes:	*Milligrams*	*Milligrams*
5	5.0×10^{-1}	5.0×10^{-1}
5	5.0×10^{-1}	5.0×10^{-1}
Days:		
1	2.5×10^{-1}	3.0×10^{-1}
1	2.5×10^{-1}	2.6×10^{-1}
2	2.0×10^{-1}	2.7×10^{-1}
3	1.3×10^{-1}	1.1×10^{-1}
4	5.7×10^{-2}	8.2×10^{-2}

BIOLOGIC CONFIRMATIONS

The following experiments (4, 5, and 6) were performed as biologic confirmations of the absorption spectrum analyses. The results are summarized in table 3.

Experiment 4

Six strain A and six strain C57 Black mice were injected intravenously with 0.5 mg. of methylcholanthrene. Thirty minutes later the lungs were removed separately, minced with a pair of scissors, and the minced tissue was injected subcutaneously into male mice of strain C3H. Each mouse received approximately one pair of minced lungs. The animals were inspected every 2 weeks for 10 months for the appearance of tumors at the site of injection.

Experiment 5

Thirty hybrid mice were injected intravenously with 0.5 mg. of methylcholanthrene. Ten mice were killed in 30 minutes, 10 in 3 days, and the remainder 7 days after injection. The lungs were

TABLE 3.—*Induction of sarcomas in strain C3H male mice following subcutaneous injection of tissue or tissue extract from mice injected intravenously with 0.5 mg. methylcholanthrene*

Experiment No.	Inoculum data					C3H mice injected	Tumors	Latent time
	Strain of injected mouse	Mice used	Tissue used	Time after injection	Preparation of tissue			
		Number		*Minutes*		*Number*	*Number*	*Weeks*
4	A	6	Lung	30	Minced	4	2	12
	C57 Black	6	do	30	do	4	3	15
	Hybrid	10	do	30	Ether extract	3	3	10
				Days				
5	do	10	do	3	do	3	1	16
	do	10	do	7	do	3	0	
6	A	3	Whole mouse	10	do	3	0	
	C57 Black	3	do	10	do	3	0	
	A	3	do	20	do	3	0	
	C57 Black	3	do	20	do	3	0	

removed, minced, dried *in vacuo* over anhydrous phosphoric pentoxide, and extracted with ether in a Sohxlet apparatus.

Each of the three extracts was mixed with filtered lard to bring the volume to 0.9 cc., and 0.3 cc. was injected subcutaneously into male mice of the C3H strain. The animals were observed for tumors for 10 months.

Experiment 6

Six strain A and six strain C57 Black males were injected intravenously with 0.5 mg. of methylcholanthrene. Three mice of each strain were killed 10 days, and three of each strain were killed 20 days later. The carcasses were ground, dried *in vacuo* over anhydrous phosphoric pentoxide, and extracted with ether in a Sohxlet apparatus.

The resultant extracts, freed of ether, were injected into C3H male mice, 0.25 cc. every 2 weeks, until the extracts were exhausted. The animals were inspected for tumors during the following 12 months.

RESULTS AND DISCUSSION

Figure 2 shows the disappearance of ether-extractable 20-methylcholanthrene from the lungs and whole bodies of mice, plotted semilogarithmically from the data of tables 1 and 2. The rates of disappearance are shown schematically by the straight lines. Due to interfering substances in the whole body extracts, it was not possible to extend the curve beyond the fourth day. During that time, however, it is approximately parallel to the curve of disappearance from the lungs.

The lines are represented by the following formula:

$$A = A_0\, 10^{-kt}$$

where A_0 is amount of hydrocarbon present at time o; A, the amount of hydrocarbon present at time t; t, time after injection, in days; and k, the disappearance constant. For the lungs, k is 0.27, i. e., in approximately 4 days the amount of methylcholanthrene in the lungs diminished to one-tenth of its original value.

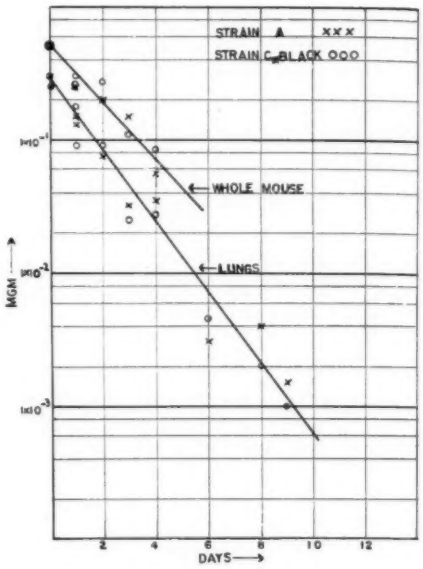

FIGURE 2.—Disappearance of ether-extractable methylcholanthrene in mice following single intravenous injection of 0.5 mg. of the hydrocarbon.

In all these determinations only absorption bands belonging to unchanged methylcholanthrene were observed. No spectroscopic evidence of a chemical change of the hydrocarbon was found.

In the curves the experimental points for strain A mice and strain C57 Black mice show no significant or consistent divergence. Despite the sharp difference in susceptibility between these strains the disappearance of methylcholanthrene from the lungs and the whole bodies is the same in the two strains. It is evident, therefore, that difference in the rate of elimination of methylcholanthrene is not the explanation

for the difference in the susceptibility to pulmonary tumors in these two strains of inbred mice.

The biologic data confirm the absorption spectrum analyses. Tumors arose at the site of injection in C3H mice receiving minced lung or extract of lungs from animals that were injected with methylcholanthrene 30 minutes previously. Extracts of 10 lungs from mice injected 3 days previously also produced 1 tumor, but extracts of lungs 7 days after the methylcholanthrene administration did not.

None of the animals injected with extracts of carcasses of mice which had received methylcholanthrene 10 or 20 days previously developed tumors. Methylcholanthrene dissolved in ethyl ether extract of mouse carcasses (0.5 mg. per 0.25 cc.) readily produces sarcomas at the site of injection (*12*).

The results indicate that intravenously injected methylcholanthrene detectable by the method used disappears rapidly from the lungs and the whole body of the mouse. It is possible, of course, that a conversion or conjugation product of methylcholanthrene, not extractable with ether or detectable by the absorption spectrum, is the active agent involved in the induction of pulmonary tumors in mice. If it is assumed that methylcholanthrene itself is the active agent, however, the tumors are produced either by extremely small quantities of the chemical that are retained by the tissues and exert constant action over a protracted period, or the action of the chemical is brief, initiating an irreversible series of reactions that eventuate in carcinogenesis.

Pulmonary tumors in strain A mice are induced within 6 weeks after the intravenous administration of 0.5 mg. of methylcholanthrene; in strain C57 Black mice, the reaction becomes manifested in 6 to 8 months (*5*). It therefore seems probable that pulmonary tumors arise long after the introduction of the stimulus and long after the removal of the stimulus from the body.

CONCLUSION

Following the intravenous injection of 20-methylcholanthrene, the ether-extractable hydrocarbon disappears at the same rate from the lungs and the whole bodies of mice of strain A (highly susceptible to pulmonary tumors) as from the lungs and the whole bodies of mice of strain C57 Black (resistant to pulmonary tumors).

The rate of disappearance of methylcholanthrene, therefore, does not explain the marked difference in the susceptibility of the two strains of mice to induced primary pulmonary tumors.

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Factors Influencing the Induction of Pulmonary Tumors in Strain A Mice by Carcinogenic Hydrocarbons

By MICHAEL B. SHIMKIN, *assistant surgeon*, and EGON LORENZ, *senior biophysicist, National Cancer Institute, National Institute of Health, United States Public Health Service*

Previous communications[1] from the National Cancer Institute reported the induction of primary pulmonary tumors in mice of strain A with 10 carcinogenic agents and described the effect of certain factors upon the reaction, particularly the dose, the route of injection, and the medium in which the hydrocarbon was dispersed or dissolved. It was reported that the reaction could be used as a quantitative test of the carcinogenic potency of a wide variety of chemicals, and that there was no constant quantitative or qualitative correlation between the carcinogenicity of a compound for the lungs and for the subcutaneous or the cutaneous tissues of mice.

The present communication reports investigations of several additional factors which could influence the induction of pulmonary tumors in strain A mice. These factors are: (1) The effect of particle size of the intravenously injected hydrocarbons; (2) the effect of the age and weight of mice; (3) the effect of repeated as contrasted with single intravenous injections; (4) the effect of foster nursing; and (5) the effect of reticuloendothelial blockade. One of the main objectives of the studies was to obtain information regarding the mechanism of the response, particularly whether it is a local effect of the carcinogen on the pulmonary tissue or a manifestation of a general effect on the whole organism.

[1] *For references, see Shimkin* (1) *and Andervont and Shimkin* (2).

EXPERIMENTAL PROCEDURE

DISPERSIONS OF HYDROCARBONS

The carcinogens employed in these experiments were 20-methylcholanthrene (m. p. 179.8°–180.4° C., corrected) and 1,2,5,6-dibenzanthracene (m. p. 266.0°–266.8° C., corrected).[2]

To prepare the different types of dispersions of these hydrocarbons for intravenous injection two methods (*3–6*) were used:

Dispersions in aqueous cholesterol colloid

One gram of cholesterol dissolved in 100 cc. of ethyl ether was added to 1 liter of a 0.01-percent solution of the dioctyl ester of sulfosuccinic acid (Aerosol O. T.)[3] in distilled water. The ether was blown off under violent agitation, first at a temperature of 0° to 4° C., and finally at room temperature. A stable dispersion of cholesterol in water was obtained after filtration through two layers of finely textured filter paper. With the aid of this cholesterol colloid, which does not settle out after long standing, the following dispersions of hydrocarbons were prepared.

Dispersion 1 (fine).—To 196 cc. of the cholesterol colloid was added 40 mg. of methylcholanthrene dissolved in an excess of ethyl ether (e. g., 100 cc.). The ether was blown off as described above, and during the process 4 cc. of a 1-percent

[2] *Hereinafter referred to as methylcholanthrene and dibenzanthracene, respectively.*

[3] *Hereinafter referred to as Aerosol.*

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solution of Aerosol in water was added slowly.

The particles of the hydrocarbon in this dispersion, as measured with an ocular micrometer, were mainly round and 1.7μ to 4.0μ in diameter (average approximately 2.5$\mu\times$2.5μ). A few long needles, 50.0$\mu\times$0.8μ to 1.0μ, were also present.

Dispersion 2 (fine).—Aerosol was added to the cholesterol colloid to give an Aerosol concentration of 0.03 percent. To 200 cc. of this colloid was added 40 mg. of methylcholanthrene dissolved in an excess of ether. The ether was blown off as described above.

The methylcholanthrene particles of this dispersion were round and measured 0.6μ to 1.5μ in diameter (average 1.0$\mu\times$1.0μ); no needles were present.

Dispersion 3 (coarse).—Twenty milligrams of crystalline methylcholanthrene was ground in a glass mortar with the addition of 0.4 cc. of a 5-percent aqueous Aerosol solution. To the resultant paste was added 100 cc. of the cholesterol-Aerosol colloid.

Over 60 percent of the particles in this suspension were irregular masses measuring 10$\mu\times$10μ to 45$\mu\times$10μ. Some large needles measuring 50μ to 80$\mu\times$2μ to 3μ were also present. The average size of the particles was estimated to be approximately 25$\mu\times$10μ.

Dispersion 4 (coarse).—Two cubic centimeters of a previously prepared dispersion of methylcholanthrene in horse serum, containing 40 mg. of the hydrocarbon, was diluted with 10 cc. of an 0.85-percent solution of sodium chloride and shaken. After standing for 2 hours, the supernatant fluid containing the finest particles was poured off. Another 10 cc. of saline was added, the suspension shaken, and after 15 minutes the supernatant fluid poured off. The remaining particles were resuspended in saline and filtered through coarse filter paper, then washed with saline and finally with distilled water. After drying the filter paper at 120° C. for 15 minutes, 16.0 mg. of coarse particles was obtained. To these 16.0 mg., 80 cc. of the cholesterol colloid was added and its Aerosol concentration adjusted to 0.03 percent. Shaking the suspension with glass beads broke up the clumps that were present.

About one-third of the particles of this dispersion were round and measured approximately 2.5μ in diameter. The remainder consisted of large irregular masses, 7$\mu\times$15μ to 10$\mu\times$20μ, and some large needles measuring 50μ to 80$\mu\times$2μ to 3μ. The average size of the particles was estimated to be approximately 15$\mu\times$10μ.

Dispersions in horse serum-cholesterol

To 1 liter of fresh horse serum was added 5 cc. of a 1-percent aqueous solution of Aerosol to produce a concentration of 0.005 percent. A solution of cholesterol in ethyl ether (1 gm. in approximately 200 cc.) was then added and the mixture shaken briefly. The ether was blown off with air at 38° to 40° C., and the excess cholesterol was removed by filtration under suction through two layers of fine-textured filter paper. One gram of hydrocarbon was then dissolved in an excess of ethyl ether, and this solution added to the horse serum-cholesterol dispersion. After brief shaking the ether was blown off with nitrogen, under violent agitation, at a temperature of 0° to 4°. When most of the ether had been removed, the temperature of the dispersion was raised slowly to room temperature. Two fairly uniform dispersions were thus prepared:

Dispersion 5 (methylcholanthrene).—The particles in dispersion 5 were between 1$\mu\times$1μ and 6$\mu\times$6μ in size, the majority being approximately 2$\mu\times$3μ.

Dispersion 6 (dibenzanthracene).—The particles in dispersion 6 consisted of needles measuring from $0.5\mu \times 10\mu$ to $2\mu \times 18\mu$; the average size of the masses was estimated to be approximately $1\mu \times 15\mu$.

All four dispersions of methylcholanthrene in aqueous cholesterol colloid contained 0.2 mg. of the hydrocarbon per cubic centimeter and 0.03 percent of Aerosol. The two horse serum-cholesterol dispersions contained 1 mg. of the hydrocarbon per cubic centimeter and 0.005 percent of Aerosol. The hydrocarbon content was checked in each case by absorption spectrum analysis of the ether extract.

The dispersions were stored at approximately 4° C. and remained suitable for intravenous injection into mice for over a year after preparation. Dispersion 2 remained in suspension during the year. A small part of dispersion 1 and all of the other four dispersions, however, settled out on standing; resuspension was easily accomplished by shaking.

Experimental Animals

The dispersions of the hydrocarbons were injected intravenously into a lateral tail vein of strain A mice. The strain A mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. They were maintained on a diet of Purina dog-chow pellets[4] and had access to an unlimited supply of water. The animals were 2 to 3 months of age at the beginning of the experimental procedures unless otherwise specified. The experimental groups included approximately equal numbers of males and females which were kept in separate wooden boxes with sawdust as bedding.

At designated periods after the injection of the carcinogens the animals were killed by cervical dislocation and the lungs fixed in Tellyesniczky's fluid. After fixation, the lungs were examined for the presence and the number of white subpleural nodules. Since the gross appearance of these lesions is typical (*1*, 2) and the histologic appearance has been described fully elsewhere (*1*, *7*), only a few representative sections were taken for histologic verification.

In previous reports (*1*, *2*) only the lungs that contained pulmonary nodules were included in determining the average number of pulmonary tumors induced; the average was multiplied by the percent of mice that developed pulmonary tumors. In the present report, the total number of pulmonary tumors in each group of animals is divided by the number of animals used, to obtain the mean value for the number of pulmonary tumors in the group.

Experiment 1, Effect of Particle Size

Methylcholanthrene dispersions 1 and 2 (fine particles) were injected into 57 and 40 strain A mice, respectively, and dispersions 3 and 4 (coarse particles) were injected into 55 and 40 mice, respectively. All mice received a single intravenous administration of 0.5 cc., containing 0.1 mg. of methylcholanthrene. This dose was tolerated by all the animals.

The mice were killed 8, 12, and 18 weeks after the injection of the hydrocarbon, and their lungs examined for the presence and number of pulmonary tumors.

Results

The results are presented in table 1. There is a large and obvious difference in the number of pulmonary tumors induced: the mice injected with the dispersions containing coarse particles had

[4] *According to the manufacturer, the chow contains 20 percent protein, 3 percent fat, 56 percent carbohydrate, 6 percent ash, and 15 percent water, with vitamins A and G added.*

over 10 times as many pulmonary nodules as those that received dispersions of fine particles. By applying Student's test to the difference of the mean values of the number of pulmonary tumors per mouse for the fine and coarse dispersions, the probability is found to be < 0.01, indicating the high significance of the results. The differences of the mean values of the number of tumors between dispersions 1 and 2 and between dispersions 3 and 4 are also significant ($P < 0.01$ and $P \leqq 0.05$, respectively).

The greater reaction of the lungs to large particles of methylcholanthrene as contrasted with the effect of fine particles suggested that the amount of hydrocarbon deposited in the lungs is of prime importance. It was thought that the large particles, carried by the blood stream into the pulmonary circulation, were unable to pass through the capillary system and were caught in the pulmonary tissue, whereas the small particles passed through the lungs and were distributed in the general circulation. This interpretation was substantiated by absorption spectrum analysis of the lungs of mice following intravenous injection of the dispersions.

Thirty strain A mice were injected intravenously, each with 0.5 cc. of one of the dispersions 1, 2, and 4 containing 0.1 mg. of methylcholanthrene. Twelve mice received dispersion 1, 6 mice dispersion 2, and 12 mice dispersion 4. The lungs were removed 30 minutes, 24, and 48 hours after injection and extracted with ether. The method of extraction and the determination of the amount of ether extractable hydrocarbon by absorption spectrum analysis were described previously (*8*). In some cases extracts of lungs of a single mouse, in others extracts of lungs from 2 mice were used. The results are summarized in table 2. The values of the table are mean values of 2 determinations except for dispersion 4 which is the mean value of 6 determinations. For lungs removed 30 minutes after injection, these mean values lie within the limits of variation, determined previously (*8*) i. e., ± 21 percent. For lungs removed after 24 and 48 hours, this error increases as the ether extractable hydrocarbon content decreases and becomes $= \pm 50$ percent or more near the limit of sensitivity of the method (5×10^{-4} mg. of methylcholanthrene per cubic centimeter if a 1-cm. absorption cell is used).

Table 2 shows that with dispersion 4, containing large particles of methylcholanthrene, over 60 percent of the intravenously injected compound was recovered

TABLE 1.—*Effect of particle size on induction of pulmonary tumors with 0.1 mg. methylcholanthrene dispersed in aqueous cholesterol colloid (experiment 1)*

Dispersion No.	Particle size	Time after injection	Mice injected	Mice with pulmonary tumors, according to number of pulmonary tumors per mouse										Average number of pulmonary tumors per mouse	Standard deviation
				0	1	2	3-5	6-10	11-20	21-30	31-40	41-50	51		
	Micra	*Weeks*	*Number*												
2	1.0×1.0	12	40	19	12	6	3	.....	.....	.....	.....	.....	.....	0.85	1.01
1	2.5×2.5	8	10	3	2	4	1	.....	.....	.....	.....	.....	.....	1.5	1.43
1	2.5×2.5	12	37	4	5	13	14	1	.....	.....	.....	.....	.....	2.5	1.80
1	2.5×2.5	18	10	.....	2	3	4	.....	1	.....	.....	.....	.....	3.9	4.47
4	15×10	12	39	.....	.....	1	.....	3	13	9	9	1	3	25.7	20.22
3	25×10	8	10	.....	.....	.....	2	5	3	.....	.....	.....	.....	9.9	4.58
3	25×10	12	35	.....	.....	.....	1	1	5	10	7	8	3	34.2	15.80
3	25×10	18	10	.....	.....	.....	.....	.....	1	4	2	2	1	34.1	13.34

TABLE 2.—*Methylcholanthrene content of lungs of mice injected intravenously with 0.1 mg. methylcholanthrene as fine or coarse dispersions in aqueous cholesterol colloid (experiment 1)*

Dispersion No.	Particle size	Methylcholanthrene content per mouse according to the time after injection		
		30 minutes	24 hours	48 hours
	Microns	*Milligrams*	*Milligrams*	*Milligrams*
4	15×10	6.6×10^{-2}	2×10^{-2}	$\sim 7 \times 10^{-3}$
1	2.5×2.5	1.0×10^{-2}		$\sim 1 \times 10^{-3}$
2	1.0×1.0	$\ll 1 \times 10^{-2}$	$\ll 1 \times 10^{-3}$	[1] $\ll 1 \times 10^{-3}$

[1] No methylcholanthrene was detected; the value given corresponds to the limit of sensitivity of the method for the volume of extract used.

from the lungs 30 minutes after injection. With dispersion 1, containing small particles and some long needles of the hydrocarbon, only 10 percent of the amount injected became lodged in the lungs. With dispersion 2, consisting of particles measuring up to 1.5μ in diameter, the amount that became lodged in the lungs was below the limit of sensitivity of the method for methylcholanthrene.

The number of pulmonary tumors induced in strain A mice following intravenous injection of methylcholanthrene appears, therefore, to be primarily dependent upon the dose retained by the lungs, rather than upon the dose injected into and distributed elsewhere in the body. Previous experiments (*9*), in which the introduction of nonspecific irritants into the lungs did not increase the neoplastic reaction to methylcholanthrene, indicate that the greater irritation produced by the larger particles of the coarse dispersions is a factor of minor importance.

EXPERIMENT 2, EFFECT OF AGE AND WEIGHT OF MICE

Two groups of strain A mice were used: 60 mice 11 to 12 months of age, weighing an average of 32 gm., whose lungs weighed between 0.28 and 0.30 gm.; and 70 mice 2 months old, weighing an average of 21 gm., whose lungs weighed between 0.16 and 0.17 gm.

The mice were given a single intravenous injection of dispersion 6, diluted with the vehicle to equal 0.1 mg. of dibenzanthracene per 0.25 cc. They were killed 12 weeks after injection and their lungs examined for the presence and the number of pulmonary nodules.

Results

The results are summarized in table 3. The old mice developed approximately half the number of pulmonary tumors that were obtained in the young mice. By applying Student's test to the difference of the mean values, the result is found to be highly significant ($P < 0.01$). The difference is increased if correction is made for the expected incidence of spontaneous pulmonary tumors in the two groups at the respective ages of 14–15 and 5 months: 70 percent for the older groups and 10 percent for the younger animals (*1*).

There are three known variates in the two groups of experimental animals: age, body weight, and the weight of the lungs. It is possible that old pulmonary tissue is more resistant to the action of dibenzanthracene, or that the difference in response was owing to the difference in the dose per body weight administered. It was shown in the preceding experiment, however, that the pulmonary reaction seems to depend primarily upon the dose of the hydrocarbon that reaches and is retained in the lungs.

To obtain data on the amount of dibenzanthracene that was deposited in the lungs, two mice of each of the two groups were injected intravenously with 0.1 mg. of dibenzanthracene. The animals were killed after 30 minutes and the lungs extracted with ether. Absorption spec-

TABLE 3.—*Effect of age and weight of mice upon induction of pulmonary tumors with 0.1 mg. dibenzanthracene in horse serum-cholesterol colloid, 12 weeks after injection (experiment 2)*

Age of mice (months)	Body weight	Lung weight	Mice injected	Mice with pulmonary tumors, according to number of pulmonary tumors per mouse							Average number of pulmonary tumors per mouse	Standard deviation
				0	1	2	3–5	6–10	11–20	21–30		
	Grams	*Grams*	*Number*									
11–12	32	0.29	57	4	11	12	15	12	3	.....	3.8	3.39
2	21	.16	70	1	1	5	21	26	15	1	8.1	4.96

trum analysis revealed that the same amount of dibenzanthracene, 4.5×10^{-2} mg., was present in the lungs of the older as well as in the younger mice.[5] Since the lungs of the older mice were about twice the weight of the lungs of the younger mice at the time of injection, however, the concentration of the hydrocarbon in the tissue was considerably higher in the lungs of the younger mice.

It seems probable that the major factor involved in the induction of the greater number of pulmonary tumors in the younger animals is the higher concentration of dibenzanthracene obtained in their lungs, because of the smaller size and weight of the lungs as compared with the lungs of the older, larger animals.

EXPERIMENT 3, EFFECT OF REPEATED INJECTIONS

Data of spectroscopic analyses, reported in the preceding paper (*8*), indicated that the ether-extractable carcinogenic hydrocarbons injected intravenously as dispersions disappear rapidly from the lungs of mice. The initial concentration of the hydrocarbon, therefore, is maintained in the lungs for a relatively short time.

It was of interest to ascertain the importance of the factors of concentration level and time in contrast with that of the total dose of the carcinogen. This could be shown by comparing the effect of single doses of the carcinogen with the effect produced by dividing the dose into several repeated administrations.

Twenty strain A mice were injected with 0.5 cc. of dispersion 2, containing 0.1 mg. of methylcholanthrene. The total amount was given in a single intravenous administration. Twenty other mice received the same dose in five intravenous injections of 0.1 cc. at 3-day intervals. Thirty mice of the same age were kept as untreated controls. The animals were killed 20 weeks after injection and their lungs examined for pulmonary tumors.

Another experiment was performed with dispersion 5, diluted with horse serum-cholesterol colloid so that the concentration of methylcholanthrene was 0.2 mg. per cubic centimeter. Twenty strain A mice received a single intravenous injection of 0.4 cc. (0.08 mg.). Forty mice were given 4 intravenous injections of 0.1 cc. of the dispersion at 3-day intervals. These animals tolerated 3 injections without mortality, but a few hours after the fourth administration 18 died with symptoms of anaphylactic shock. The surviving mice of this series were killed 14 weeks after injection and the lungs examined for pulmonary tumors.

Results

The results are presented in table 4. It is evident that the incidence and the mean number of pulmonary tumors were the same in each case where comparable

[5] *The values of the four determinations lie well within the limits of variation of the method, ± 21 percent* (8).

TABLE 4.—*Effect of repeated injections on induction of pulmonary tumors with methylcholanthrene (experiment 3)*

Dispersion No.	Total dose	Injections	Time after injection	Mice injected	Mice with pulmonary tumors, according to number of pulmonary tumors				Average number of pulmonary tumors per mouse
					0	1	2	3-5	
	Mg.	*Number*	*Weeks*	*Number*					
2	0.1	1	20	20	6	9	4	1	1.05
2	.1	[1] 5	20	20	7	9	2	2	1.00
5	.08	1	14	19	9	6	3	1	.79
5	.08	[1] 4	14	18	9	6	2	1	.72

[1] At 3-day intervals.

doses of methylcholanthrene were given either in single injections or in repeated injections.

Single intravenous injections of methylcholanthrene, therefore, are as efficacious in producing pulmonary tumors as five repeated injections given at 3-day intervals. Under the conditions of the experiment, the main factor in the induction of pulmonary tumors in mice appears to be the total dose of the hydrocarbon that is administered and that reaches and lodges in the lungs.

EXPERIMENT 4, EFFECT OF FOSTER NURSING

Bittner (*10*) indicated that foster nursing of strain A mice (susceptible to pulmonary and to mammary tumors) by C57 Black mothers (resistant to pulmonary and to mammary tumors) does not influence the occurrence of spontaneous pulmonary tumors in strain A mice. This observation was tested in a study of induced pulmonary tumors.

Thirty strain A males and females, offspring of strain A females that had been foster nursed by C57 Black mice, were obtained from Dr. G. B. Mider. These mice and 30 strain A mice that were nursed by their own mothers were injected intravenously with 0.1 mg. of dibenzanthracene in 0.1 cc. of horse serum-cholesterol colloid (dispersion 6). The animals were killed 8 and 14 weeks following the administration of the hydrocarbon, and their lungs examined for the presence and the number of pulmonary tumors.

Results

The results are summarized in table 5. The observed differences of the mean values of the number of pulmonary tumors agree within the limits of chance variation.

Foster nursing, which reduces the occurrence of mammary carcinoma in strain A females (*10*), has no influence, therefore, upon the susceptibility of the mice to the induction of primary pulmonary tumors with dibenzanthracene.

TABLE 5.—*Effect of foster nursing on induction of pulmonary tumors in strain A mice with 0.1 mg. dibenzanthracene intravenously (experiment 4)*

Mice	Time after injection	Mice injected	Mice with pulmonary tumors, according to number of pulmonary tumors					Average number of pulmonary tumors per mouse	Standard deviation
			2	3-5	6-10	11-20	21-30		
	Weeks	*Number*							
Strain A fostered by C57 Black (offspring)	8	10	1	6	2	1		5.6	4.40
Do	14	19			5	11	3	14.5	5.45
Normal	8	15	4	4	4	3		6.9	5.11
Do	14	15			3	11	1	13.6	4.33

Experiment 5, Effect of Reticulo-Endothelial Block

Blockade of the reticulo-endothelial system with trypan blue reduces the immunologic resistance of mice to transplantable tumors (*11*). To ascertain whether this procedure would influence the induction of pulmonary tumors with carcinogenic hydrocarbons, 30 strain A mice were injected intraperitoneally with 0.2 cc. of a 1.5-percent aqueous solution of trypan blue. The injections were repeated twice during the following 2 days. The mice were then injected intravenously with 0.25 mg. of dibenzanthracene in 0.25 cc. of horse serum-cholesterol colloid (dispersion 6). Another intraperitoneal injection of trypan blue was given a week following the administration of dibenzanthracene. Twenty normal mice were injected intravenously with 0.25 mg. of dibenzanthracene (dispersion 6) for comparison.

The mice were killed 14 and 20 weeks following injection of the hydrocarbon, and the lungs examined for the presence and the number of pulmonary nodules. The skin and the visceral organs of the mice that received trypan blue remained blue until the termination of the experiment.

Results

The results are presented in table 6. The mean numbers of pulmonary tumors in the mice that received trypan blue are in agreement within the limits of chance variation with the mean numbers of pulmonary tumors in the mice that were injected with dibenzanthracene only. Grady [6] also found that injection of trypan blue had no effect on the incidence of pulmonary tumors induced in strain A mice by subcutaneous injection of methylcholanthrene.

DISCUSSION

The mechanism of induction of pulmonary tumors in mice can be divided into two main phases: (1) The mode of action of the carcinogen; and (2) the mode of response of the organism to the stimulus. The present status of the problem does not permit final conclusions; on the other hand, there is some experimental evidence to indicate the likely possibilities.

Mode of Action of the Carcinogen

The appearance of pulmonary tumors in mice injected with carcinogenic hydrocarbons can be ascribed either to the local action of the carcinogens on the pulmonary tissue, to a general systemic action of the compound resulting in a lowered resistance to tumor development, or to a combination of the two.

The most efficacious method of inducing primary pulmonary tumors in mice, both from the standpoint of the shortest latent time and the greatest response as measured

[6] *Grady, H. G.: Unpublished data.*

Table 6.—*Effect of reticulo-endothelial blockade on the induction of pulmonary tumors with 0.25 mg. of dibenzanthracene intravenously (experiment 5)*

Mice	Time after injection	Mice injected	Mice with pulmonary tumors, according to number of pulmonary tumors					Average number of pulmonary tumors per mouse	Standard deviation
			11–20	21–30	31–40	41–50	51		
	Weeks	*Number*							
Injected with trypan blue	14	20	2	7	6	4	1	32.7	10.52
Do	20	10	1	1	3	2	3	42.4	14.32
Normal	14	10	.....	4	3	3	.....	35.3	8.94
Do	20	10	.....	.....	5	2	3	45.8	15.06

by the number of discrete tumors, is the intravenous injection of carcinogenic hydrocarbons. This route produces the maximum contact of the carcinogen with the lungs. Pulmonary tumors are also induced when silk threads coated with dibenzanthracene are placed in the lungs of mice, or when dispersions of the compound are introduced into the trachea (*1*, *12*). After subcutaneous or oral administration of large amounts of dibenzanthracene the absorption spectrum fails to reveal the presence of the compound or its derivatives in the lungs, although multiple pulmonary tumors are induced. That the agent or that some photoactive derivatives reach the lungs is suggested, however, by the presence of photodynamic activity in suspensions of lungs of mice injected subcutaneously with 3, 4-benzpyrene (*13*).

Important support to the belief that actual contact of the carcinogen and the lungs is necessary is furnished by the experiments with charcoal-adsorbed dibenzanthracene (*14*). Pulmonary tumors are not produced by this preparation when it is injected subcutaneously, although sarcomas at the site of injection are induced; upon intravenous administration, the preparation induces pulmonary tumors. Further investigations indicated that more pulmonary tumors are evoked when the compound is injected in a medium that leaves the site of subcutaneous injection than in menstrua that are retained at the site of injection (*4*). Moreover, it was shown (*2*) that there is no constant correlation between the carcinogenicity of a compound for the lungs and for the subcutaneous or cutaneous tissues of mice. Since certain compounds appear to be selective in their effect on certain tissues, the response is probably not a general one involving the whole organism.

The first experiment recorded here offers strong support to the conclusion that pulmonary tumors are the result of direct action of the carcinogen on the pulmonary tissue. Two groups of mice were injected intravenously with 0.1 mg. of methylcholanthrene. In 1 group, owing to the presence of large particles in the dispersion, 60 percent of the compound became lodged in the lungs, and the animals developed an average of 25 pulmonary tumors. In another group, most of the particles of the dispersion were sufficiently small to pass through the pulmonary capillaries and into the general circulation. The lungs retained 10 percent of the carcinogen, and the animals developed an average of 2.5 tumors of the lung. It is evident that the number of pulmonary tumors induced in strain A mice is a function of the dose of the carcinogenic hydrocarbon that reaches and lodges in the lungs, rather than of the dose injected into the whole organism.

In the second experiment, a group of young, small mice developed twice the number of pulmonary tumors than did older, larger mice. An equal amount of dibenzanthracene was injected intravenously and an equal amount was retained in the lungs of both groups. The results can be interpreted as being due to the difference in age or the dose per total body weight administered. In view of the evidence of the first experiment, however, it is probable that the difference in the number of pulmonary tumors is due primarily to the higher concentration of the carcinogen in the smaller lungs of the younger mice.

It is reported elsewhere (*8*) that following intravenous injection, the amount of hydrocarbon spectroscopically detectable in extracts of lungs diminishes rapidly. In 4 days, approximately one-tenth of the amount that became lodged in the lungs after injection was detected by means of absorption spectrum analysis. Unless it is

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assumed that a conversion or conjugation product that is not extractable with ether or detectable by the absorption spectrum is the active agent, two possibilities can be considered in the mode of action of the chemicals in inducing pulmonary tumors by the intravenous route: either that extremely small quantities of the chemical are retained by the tissues and exert constant action over a protracted period, or that the action of the chemicals is a brief one initiating an irreversible series of reactions and continuing to carcinogenesis without the necessity of further stimulus by the carcinogen.

The second possibility is supported by the third experiment presented here. If the reaction were due to constant action over a considerable length of time, it might be expected that the augmentation of the length of action by means of repeated injections would produce more pulmonary tumors than the same dose given in a single injection. Actually, the same number of tumors was elicited when the dose was divided into five injections at 3-day intervals as when the compound was given in one dose.

Mode of Reaction of the Lung

The susceptibility of mice of various inbred strains to induced pulmonary tumors is parallel to the susceptibility of the strains to the spontaneous development of this neoplasm. Moreover, the histologic appearance of the induced pulmonary tumors is indistinguishable from the structure of the spontaneous growths. It was stated previously (*15*) that this suggests that the carcinogenic hydrocarbons are accelerators of some process inherent in the animal. This acceleration resembles in some ways the acceleration in the appearance of mammary tumors in mice that receive large doses of estrogens. The estrogens, however, must be given over a protracted period of time (*16*), and it has been indicated (*17*) that the stimulus merely prepares a suitable substratum for the action of another agent, transmitted through the mother's milk, which leads to the development of mammary carcinoma.

The postulation of a hypothetical unknown agent in the animal body which induces pulmonary tumors in mice through stimulation by the carcinogenic hydrocarbons cannot be ignored in view of the fundamental clarification of our knowledge of mammary tumors by the demonstration of the milk factor. Andervont (*12*) also considered this possibility and drew the analogy between the reaction in the lungs of mice following the administration of carcinogenic hydrocarbons and the localization of intravenously injected Shope papilloma virus in the tarred ears of rabbits.

There is no experimental evidence that an unknown agent is involved in the development of pulmonary tumors in mice. It has been shown that nonspecific chronic irritation in the lungs does not enhance the action of the hydrocarbons in producing pulmonary tumors (*9*). The fourth experiment presented in this report shows that the milk factor, essential for the genesis of mammary cancer in mice, is not involved in the induction of pulmonary tumors by carcinogenic hydrocarbons. The fifth experiment indicates that reticulo-endothelial blockade, which influences the immunologic response of mice to transplantable tumors, is not operative in the case of induced pulmonary tumors.

Andervont's investigations (*12*) have shown that the genetic constitution of mice should not be regarded as the only factor involved in the appearance of induced pulmonary tumors. The heredity factors exert their influence by controlling the degree of susceptibility. Recently Heston

(*18*) demonstrated that this susceptibility is associated with several known genes of mice.

From the evidence available at present, it is suggested that the induction of pulmonary tumors in mice by the intravenous administration of carcinogenic hydrocarbons is perhaps best explained by the following mechanism: (1) The carcinogenic hydrocarbons act directly upon the pulmonary tissue of mice; (2) the pulmonary tissue of mice is the locus of a process, present in varying degrees in all strains, that under normal conditions eventuates in the development of spontaneous pulmonary tumors; (3) the carcinogen accelerates this process, both in regard to the time of appearance of the tumors and in the number of discrete sites of carcinogenesis; and (4) the acceleration of the process is rapid and apparently the increased pace continues without further stimulation by the chemical agent.

Pulmonary-Tumor-Induction Test of Carcinogenic Potency

A previous report (*2*) suggested that the intravenous injection of dispersions of carcinogenic hydrocarbons and other compounds into strain A mice can be used as a method of biologic evaluation of carcinogenic potency. It was pointed out that the dose, the medium in which the compounds are introduced, and the time after administration influence the number of pulmonary tumors elicited.

This communication adds two additional factors that influence the reaction, namely, the size of the particles in the dispersion and the size of the lungs into which they are injected. Both factors, not known previously, are manifestations of the concentration of the carcinogen in the lungs. The radical differences in the number of pulmonary tumors obtained with fine as compared with coarse dispersions of methylcholanthrene, and with lungs of mice that differed in weight by a factor of two, make it evident that unless these factors are known and controlled, quantitative comparisons of chemicals by this technique cannot be made. For comparative studies, therefore, mice of the same age and weight must be employed. Secondly, unless the particle size of the dispersions for intravenous injection can be controlled, comparative quantitative studies by this method should be based on the determination of the actual amount of the compound that reaches and is retained in the lungs, such as by means of absorption spectrum analysis. Quantitative studies with the same compound are feasible only if the same dispersion is used in all phases of the particular investigation.

CONCLUSIONS

1. Intravenous injection of methylcholanthrene dispersions in which the particles are 10μ to 20μ in size induce at least 10 times as many pulmonary tumors in strain A mice as similar dispersions in which the particles measure 1μ to 2μ. The neoplastic reaction depends upon the actual amount of the hydrocarbon that lodges in the lungs and not upon the amount injected into the organism. This is evidence supporting the view that the pulmonary tumor reaction is a local one of the hydrocarbon upon the susceptible pulmonary tissue rather than a general systemic effect.

2. The lungs of young, small strain A mice are more susceptible to the induction of pulmonary tumors with intravenous dibenzanthracene than the lungs of old, large mice of the same strain. The amount of hydrocarbon retained in the lungs is the same in both cases, but the smaller lungs of the young mice permit a higher concentration of the hydrocarbon in the tissue.

3. The pulmonary tumor reaction is not influenced by foster nursing or by reticuloendothelial blockade and is the same whether the hydrocarbon is given in a single dose or in five repeated intravenous injections at 3-day intervals.

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Distribution of Acid and Alkaline Phosphatase in Tumors, Normal Tissues, and the Tissues of Tumor-Bearing Rats and Mice

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INTRODUCTION

Animal tissues are rich in enzymes which hydrolyze a wide variety of phosphate esters (*1*). These enzymes, which may be grouped under the general name of phosphatases, are active over a considerable pH range. Certain tissues possess phosphatases which are more active in the acid than in the alkaline range, while in other tissues the reverse is true. Thus, the phosphatase activity of the liver or spleen is greater at pH 4–5 than at pH 8–10, whereas the phosphatase activity of the kidney or blood serum is greater in the latter pH range (*1*). It is not known whether tissue phosphatase is a single enzyme with two or more peaks in the pH-activity curve which vary in relative magnitude from tissue to tissue, or whether it consists of a number of enzymes which function at various pH levels and whose distribution varies according to the nature of the tissue. Little or no information is available to enable one to decide between the two alternatives. For the time being, therefore, the phosphatase whose activity is determined in the acid range of pH will be referred to as acid phosphatase and, correspondingly, the phosphatase whose activity is determined in the alkaline range of pH as alkaline phosphatase, without any assumption as to the identity or lack of identity of the enzymes concerned.[1]

The activity of a number of enzymes in a wide variety of normal and of tumor tissues has been reported (*2–7*). The enzymes studied included arginase, catalase, thymonucleodepolymerase, xanthine dehydrogenase, and amylase. These studies have been extended by the investigations reported herein on the distribution of acid and alkaline phosphatase in a number of normal and tumor tissues. Emphasis is placed on the comparison of the activity of homologous tissues, i. e., tumors and the tissues from which they are derived or to which they may be related. In view of the discovery that the liver catalase activity of tumor-bearing rats (*6*) and mice (*7*) is lower than that of normal animals, the phosphatase activity of various tissues of tumor-bearing animals has also been studied. Köhler (*8*) reported that the kidney phosphatase activity increased in tumor-bearing animals during the early stages of tumor growth; it subsequently decreased with further growth of the tumor. The liver catalase activity of tumor-bearing rats follows the same course, as Greenstein, Jenrette, and White have shown (*6*).

[1] *It has been customary, in view of the marked difference in the distribution of phosphatase to refer to the acid phosphatase as spleen phosphatase and to the alkaline phosphatase as kidney phosphatase* (1). *The use of the names of specific organs to characterize a type of enzyme is likely to be confusing when the same enzyme is studied in a wide variety of tissues; hence the simple designation of acid and of alkaline phosphatase to distinguish the two types is preferred.*

EXPERIMENTAL PROCEDURE

Preparation of Tissues

Aqueous extracts of the ground tissues were prepared as described earlier (*2–7*). Tissues were removed from the animals immediately after death and ground in a glass mortar with sand. Three times the weight of tissue in distilled water was added, and the mixture allowed to stand for 18 hours at 4°–6° C. The mixture was then centrifuged and the supernatant liquid, which served as the source of the enzyme, was removed. The total N was determined on an aliquot of this supernatant. The reason for the determination of total N has been already emphasized (*6*). The enzyme studies as before were always performed on the freshly prepared extract. The animals were all fed a fox chow diet. The necrotic portions of the tumors were removed.

The tissues studied included the following transplanted tumors: rat hepatoma 31 (*2*), Jensen sarcoma in rats, mouse hepatomas (*2*), mouse sarcomas 37 and CR 180, adenocarcinoma and squamous-cell carcinoma of the stomach in mice (*9*), adenocarcinoma of the intestine in mice (*10*), adenocarcinoma of the lung in mice, malignant endothelioma of the mouse (*11*), and malignant melanoma of the mouse (*12*). In addition, a primary sarcoma in mice induced by benzpyrene and spontaneous mammary tumors in mice were also investigated.[2] The normal tissues included liver, kidney, spleen, muscle, skin, and gastric and intestinal mucosa. The mucosa was carefully removed from the stomachs and the first third of the intestines of mice of the same strain as those in which the tumors originated. In addition the sera of normal and of tumor-bearing rats were studied. Each tissue studied was obtained from three to six animals. The individual variation in such tissues was about ± 20 percent.

[2] *The tumors were supplied for these studies by the following: Rat hepatoma 31 by Dr. J. White; the Jensen rat sarcoma, mouse melanoma, mammary and intestinal tumors by Dr. J. W. Thompson; sarcomas CR 180 and 37 in mice by Dr. F. C. Turner; mouse hepatomas by Dr. H. B. Andervont; malignant mouse endothelioma by Dr. J. E. Edwards; gastric tumors in mice by Dr. H. L. Stewart; and the benzpyrene-induced sarcoma by Dr. M. J. Shear.*

Determinations of Phosphatase Activity

The substrate used was disodium phenylphosphate. For use as the substrate for acid phosphatase, 1.09 gm. of the ester was transferred to a volumetric flask of 1-liter capacity, dissolved in N/1 acetic acid-sodium acetate buffer of pH 4.6, and the flask filled to the mark with the buffer. For use as the substrate for alkaline phosphatase, 1.09 gm. of the ester together with 10.3 gm. of sodium barbiturate was transferred to a volumetric flask of 1-liter capacity, dissolved by the addition of distilled water, and more water added to bring the solution up to the mark. The pH of the latter solution was 9.5, that of the former 4.6. Both solutions were always kept in the ice box.

Five cubic centimeters of either acid or alkaline buffer substrate solution was transferred to a 15-cc. conical centrifuge tube and treated with 0.5 cc. of the enzyme solution. The mixture was shaken and allowed to incubate at 37.5° C. for 1 hour; 4.5 cc. of the Folin phenol reagent (diluted 1 : 3) was added, and the contents of the tube were stirred and centrifuged. Five cubic centimeters of the supernatant solution was transferred to a small flask and treated with 3 cc. of 20 percent sodium carbonate. After 5 minutes the colors were matched in a colorimeter against a phenol standard treated simultaneously with the test. The stand-

ard was prepared by diluting 5 cc. of a stock phenol solution (1 mg. phenol per 10 cc.) with 15 cc. of the diluted phenol reagent and 30 cc. of distilled water. Five cubic centimeters of the resulting solution, treated with 3 cc. of 20 percent sodium carbonate, served as the phenol standard. For matching the colors a glass filter which absorbed practically all radiation below 570μ was employed.

Several ratios of enzyme to substrate were employed for each tissue studied. Acid and alkaline phosphatase activity determinations were performed simultaneously with the same enzyme preparation. Appropriate blank determinations were made on the substrate and on the enzyme solutions. No spontaneous hydrolysis of the substrate in either of the buffer solutions was observed within 1 week of standing in the ice box at 5° C.

Effect of Varying the Ratio of Enzyme to Substrate

In an earlier report a method of comparison of the arginase activity of tissues by successive dilution of the extracts was described (*2*). The content of substrate remained constant throughout the series of dilutions, and the period of incubation was also the same throughout. In this way the rate of fall of enzymatic activity with dilutions of the enzyme solution provided a definite measure of the activity of the tissue from which the enzyme was derived. It was found that a rough proportionality existed between the arginase activity of all the tissues studied and the cube root of the total N concentration in milligrams per cubic centimeter of the tissue extracts.

The method of successive dilution was applied to the determination of the acid and alkaline phosphatase activity of a wide variety of animal tissues. In the case of phosphatase, the relation between activity and concentration of the extract in milligrams per cubic centimeter is not exponential, as in the case of arginase, but appears to be linear. The expression of this linear relationship is given by the symbol Q whereby

$$Q = \frac{\text{Percent hydrolysis}}{\text{Milligrams total N per cubic centimeter of tissue extract}}$$

where Q (ac) refers to acid and Q (alk) to alkaline phosphatase activity. The validity of this relation is illustrated in table 1.

TABLE 1.—*Activity of acid and alkaline phosphatase in various tissues*

[5 cc. M/200 disodium phenylphosphate incubated at pH 4.6 and 9.5 for 1 hour at 37.5° C. with 0.5 cc. extract solution]

Tissue	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Percent*		*Percent*	
Normal mouse intestinal mucosa.	0.6	20	33	100	.....
	.3	11	36	100	.....
	.03	1	33	81	2,700
	.006	0	.....	16	2,667
	.001	0	.....	3	3,000
Average	.....	.....	34	.....	2,789
Transplanted intestinal tumor in A mice.	.6	11	18	2	3
	.3	6	20	1	3
	.03	0	.....	0	.....
	.006	0	.....	0	.....
	.001	0	.....	0	.....
Average	.....	.....	19	.....	3

Tissue	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Percent*		*Percent*	
Normal mouse lung	1.2	40	33	42	35
	.24	8	33	9	37
	.12	4	33	5	41
	.03	1	33	1	33
Average	.....	.....	33	.....	36
Transplanted pulmonary adenocarcinoma in A mice.	2.9	44	15	3	1
	.58	10	17	0	.....
	.29	5	17	0	.....
	.06	1	17	0	.....
Average	.....	.....	16	.....	1

TABLE 1.—*Activity of acid and alkaline phosphatase in various tissues*—Continued

Tissue	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Per cent*		*Per cent*	
Normal mouse gastric mucosa.	.59	14	24	10	17
	.12	3	25	2	17
	.06	2	33	1	17
	.01	0	.....	0	.....
Average	.....	.....	27	.....	17
Transplanted gastric adenocarcinoma in C3H mice.	2.9	40	14	0	0
	.58	10	17	0	.....
	.29	5	17	0	.....
	.06	1	17	0	.....
Average	.....	.....	16	.....	0
Transplanted squamous-cell carcinoma of the stomach in C57 Brown mice.	2.9	18	6	10	3
	.58	4	6	2	3
	.29	2	6	0	.....
	.06	0	.....	0	.....
Average	.....	.....	6	.....	3
Normal mouse liver	4.8	58	12	16	4
	.96	12	12	4	4
	.48	6	12	2	4
	.09	1	11	0	.....
Average	.....	.....	12	.....	4
Transplanted hepatoma 1 in I × dilute brown mice.	3.5	38	11	6	1
	.7	8	11	1	1
	.35	3	9	0	.....
	.07	0	.....	0	.....
Average	.....	.....	10	.....	1
Transplanted hepatoma in C3H mice.	2.7	57	21	0	0
	.54	11	20	0	.....
	.27	6	22	0	.....
	.05	1	20	0	.....
Average	.....	.....	21	.....	0
Transplanted malignant endothelioma in C3H mice.	2.5	29	11	8	3
	.5	6	12	2	4
	.25	3	12	1	4
	.05	0	.....	0	.....
Average	.....	.....	12	.....	4
Hyperplastic mouse mammary tissue induced by stilbestrol.	1.05	18	17	9	8
	.21	4	19	2	9
	.105	2	19	1	9
	.021	0	.....	0	.....
Average	.....	.....	18	.....	9
Spontaneous mammary tumor in C3H mice.	2.6	58	22	56	21
	.5	11	22	12	24
	.25	5	20	6	24
	.05	1	20	1	20
Average	.....	.....	21	.....	22
Spontaneous mammary tumor in A mice.	2.6	55	21	50	19
	.5	10	20	10	20
	.25	5	20	5	20
	.05	1	20	1	20
Average	.....	.....	20	.....	20
Primary tumor induced by benzpyrene in A mice.	2.4	43	18	4	2
	.48	9	18	1	2
	.24	5	20	0	.....
	.05	1	20	0	.....
Average	.....	.....	19	.....	2

Tissue	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Per cent*		*Per cent*	
Transplanted malignant melanoma in dilute brown mice.	3.0	59	19	5	1
	.6	12	20	1	1
	.3	6	20	0	.....
	.06	1	17	0	.....
Average	.....	.....	19	.....	1
Transplanted sarcoma 37 in dilute brown mice.	2.4	55	23	4	2
	.48	11	23	1	2
	.24	6	25	0	.....
	.05	1	20	0	.....
Average	.....	.....	23	.....	2
Transplanted sarcoma CR 180 in dilute brown mice.	2.4	50	21	4	2
	.48	10	21	1	2
	.24	5	21	0	.....
	.05	1	21	0	.....
Average	.....	.....	21	.....	2
Normal mouse kidney	2.9	42	14	100	.....
	.58	8	14	100	.....
	.29	5	17	100	.....
	.06	1	16	58	966
	.012	0	.....	13	1,083
	.006	0	.....	7	1,166
Average	.....	.....	15	.....	1,072
Normal mouse spleen	2.2	100	.....	30	14
	.44	30	68	8	18
	.22	17	75	4	18
	.04	3	75	0	.....
Average	.....	.....	73	.....	17
Normal mouse muscle	2.2	42	19	6	2
	.44	8	18	1	2
	.22	4	18	0	.....
	.04	1	20	0	.....
Average	.....	.....	19	.....	2
Normal mouse skin	1.0	30	30	6	6
	.2	6	30	1	5
	.1	3	30	0	.....
	.02	0	.....	0	.....
Average	.....	.....	30	.....	5
Normal rat liver	4.0	94	23	16	4
	.8	20	25	3	4
	.4	11	27	2	5
	.08	2	25	0	.....
Average	.....	.....	25	.....	4
Transplanted hepatoma 31 in Osborne-Mendel rats.	2.1	100	.....	100	.....
	.42	22	52	100	.....
	.21	11	55	100	.....
	.04	2	50	25	625
	.008	0	.....	4	500
	.004	0	.....	2	500
Average	.....	.....	52	.....	542
Normal adult rabbit liver	4.2	58	14	14	3
	.84	12	14	3	3
	.42	6	14	2	4
	.08	1	13	0	.....
Average	.....	.....	14	.....	3

TABLE 1.—*Activity of acid and alkaline phosphatase in various tissues*—Continued

Tissue	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Per cent*		*Per cent*	
Fetal rabbit liver (20-days gestation).	2.6	58	22	0	0
	.52	12	23	0	.....
	.26	6	23	0	.....
	.05	1	20	0	.....
Average	.....	.....	22	.....	0
Transplanted Jensen sarcoma in Buffalo rats.	2.1	43	20	86	40
	.42	9	21	18	42
	.21	5	23	9	43
	.04	1	25	2	50
Average	.....	.....	22	.....	44
Normal rat kidney	.42	40	95	100	.....
	.21	19	91	100	.....
	.04	4	100	60	1,500
	.008	0	.....	12	1,500
	.004	0	.....	6	1,500
Average	.....	.....	95	.....	1,500
Normal rat spleen	2.2	86	39	43	20
	.44	18	41	10	22
	.22	10	45	5	22
	.044	2	45	1	22
Average	.....	.....	42	.....	21
Normal rat muscle	2.1	13	6	7	3
	.42	3	7	1	2
	.21	1	5	0	.....
	.04	0	.....	0	.....
Average	.....	.....	6	.....	2
Normal rat skin	.6	5	8	3	5
	.12	1	8	0	.....
	.06	0	.....	0	.....
	.01	0	.....	0	.....
Average	.....	.....	8	.....	5

Tissue	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Per cent*		*Per cent*	
Normal rat blood serum	9.5	4	.4	84	8
	1.9	1	.5	18	9
	.95	0	.....	9	9
	.19	0	.....	2	10
Average	.....	.....	.4	.....	9
Normal mouse lymph nodes.	3.6	100	.....	29	8
	.7	34	49	6	8
	.35	17	49	3	8
	.07	3	43	0	.....
	.035	2	56	0	.....
	.007	0	.....	0	.....
Average	.....	.....	49	.....	8
Normal mouse bone marrow.	3.2	100	.....	100	.....
	.64	16	25	17	26
	.32	8	25	9	28
	.06	1	17	1	17
	.03	0	.....	0	.....
	.006	0	.....	0	.....
Average	.....	.....	22	.....	23
Transplanted lymphoma 72942 in A mice.	9.6	100	.....	100	.....
	1.9	18	10	18	10
	.95	9	10	9	10
	.19	2	11	2	11
	.09	0	.....	0	.....
	.02	0	.....	0	.....
Average	.....	.....	10	.....	10

The data in table 1 reveal that the linear relation between phosphatase activity and concentration of the extract holds over at least a fiftyfold dilution of the extract for a large variety of animal tissues.[3] The expression *Q* may, therefore, be taken as a measure of the enzymatic activity of the tissues studied under the conditions chosen. *Q* is expressed as zero only when the percentage hydrolysis at the highest concentration of the extract is zero. Under these conditions it is assumed that the enzymatic activity is very nearly or wholly absent from the tissue concerned. Values of *Q* are not expressed in table 1 when the percentage hydrolysis is given as 100, or at lower concentrations of the extract when the hydrolysis is given as zero. In the former case, a hydrolysis of 100 percent may mean that complete splitting of the substrate was reached well before the end of the incubation period. In the latter case, when rather dilute extracts were employed, a value of zero for the percentage hydrolysis may simply mean that some splitting occurred but that the method of estimation is not sufficiently sensitive to determine it with accuracy.

[3] *Bodansky* (13) *has shown that under certain conditions the activity of phosphatase is directly proportional to the enzyme concentration.*

THE PHOSPHATASE ACTIVITY DURING TUMOR GROWTH

The data in table 1 for the phosphatase activity of the transplanted hepatic tumor

TABLE 2.—*Activity of acid and alkaline phosphatase in the transplanted rat hepatic tumor 31 at various stages of growth*

[5 cc. M/200 disodium phenylphosphate incubated at pH 4.6 and pH 9.5 for 1 hour at 37.5° C. with 0.5 cc. extract]

Age of tumor [1] (days)	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Mg. per cc.	*Per cent*		*Per cent*	
7	2.2	100		100	
	.44	24	55	100	
	.22	12	55	100	
	.044	3	68	25	568
	.008	0		4	500
	.004	0		2	500
Average			59		523
14	2.0	100		100	
	.4	21	52	100	
	.2	11	55	100	
	.04	2	50	20	500
	.008	0		4	500
	.004	0		2	500
Average			52		500
21	2.0	100		100	
	.4	28	70	100	
	.2	15	75	100	
	.04	3	75	24	600
	.008	0		5	625
	.004	0		2	500
Average			73		575
29	2.2	100		100	
	.44	20	45	100	
	.22	10	45	100	
	.04	2	50	20	500
	.008	0		4	500
	.004	0		2	500
Average			47		500
35	2.2	100		100	
	.44	20	45	100	
	.22	10	45	100	
	.04	2	50	18	450
	.008	0		4	500
	.004	0		2	500
Average			47		483
56	2.0	100		100	
	.4	24	60	100	
	.2	10	50	100	
	.04	2	50	24	600
	.008	0		5	625
	.004	0		2	500
Average			53		575

[1] Age of tumor refers to time elapsed since implantation.

31 in the rat reveal an extraordinarily high value for the alkaline phosphatase activity of this tissue. Of all the tissues studied, only the corresponding activity of intestinal mucosa and of the kidney surpass it. In order to see whether the unusually large value for the tumor alkaline phosphatase changes or remains constant with growth of the tumor, enzymatic determinations were performed on tumors removed at various stages of growth from a large number of rats. The results are given in table 2.

The data in table 2 show that the acid and alkaline phosphatase activity of the hepatoma remain approximately the same from 7 to 56 days' growth of the tumor. The variations in the values of Q are without significance, it is believed, and may be due both to individual variation between animals and to experimental error.

KINETICS OF PHOSPHATASE ACTIVITY

In order to determine the order of reaction of phosphatase in certain tissues with phenylphosphate as substrate, extracts of normal and cancerous hepatic tissues were incubated with the substrate over varying periods. The results obtained could be best described in terms of a monomolecular reaction as follows:

$$K = \frac{2.3}{Ct} \log \frac{100}{100\text{-percent hydrolysis}}$$

where C is the concentration in milligrams total N per cubic centimeter of extract and t is the period of incubation in minutes. Determinations of the reaction velocity at other than the given concentration of N in the extract were omitted in view of the data at various dilutions of the extract (table 1). The kinetic data on the hepatic tissues are given in table 3.

TABLE 3.—*Velocity of reaction of acid and alkaline phosphatase in normal and cancerous hepatic tissues*

[5 cc. M/200 disodium phosphate incubated at pH 4.6 and pH 9.5 for varying lengths of time at 37.5° C. with 0.5 cc. extract solution]

Tissue	Concentration of N in extract	Time of incubation	Hydrolysis at pH 4.6	K (acid)	Hydrolysis at pH 9.5	K (alkaline)
	Milligrams per cubic centimeter	*Minutes*	*Percent*	$\times 10^3$	*Percent*	$\times 10^3$
Normal mouse liver	0.6	30	7	4.0	2	1.1
		60	13	3.9	3	.9
		90	19	3.9	4	.9
		120	30	4.5	6	.9
Average				4.1		.9
Transplanted hepatoma 1 in I × dilute brown mice	1.1	30	12	3.9	2	.6
		60	22	3.7	3	.5
		90	34	4.0	4	.5
		120	43	4.0	6	.5
Average				3.9		.5
Normal rat liver	.84	30	11	4.6	2	.8
		60	20	4.4	3	.6
		90	30	4.6	4	.6
		120	40	4.7	6	.6
Average				4.6		.6
Transplanted hepatoma 31 in Osborne-Mendel rats	.04	30	2	17.5	13	115.0
		60	3	12.5	24	112.5
		90	4	12.5	34	112.5
		120	6	12.5	43	110.0
Average				13.7		112.5

It is clear that, at least at the concentrations of extracts given, the course of both acid and alkaline phosphatase activity follows a monomolecular reaction. In this, the phosphatase activity is similar to that found for arginase which also follows the course of a monomolecular reaction (*2–7*, *14*).

THE PHOSPHATASE ACTIVITY OF THE TISSUES OF TUMOR-BEARING ANIMALS

In earlier reports (*6*, *7*), it was stated that the liver catalase of rats and of mice bearing tumors was in general very much lower than that of normal animals. Tissues other than the liver were not extensively studied in this connection because of the relatively small amount of catalase in them. Phosphatase appears to have a more generally equal distribution throughout the animal organism than has catalase, and for this reason the phosphatase activity of a number of the tissues of tumor-bearing animals was determined and compared with that of the tissues of normal animals. The tissues selected for study were liver, kidney, spleen, muscle, and skin. The blood serum of tumor-bearing rats was also included. The tumors included the transplanted hepatic in both rats and mice, and the transplanted Jensen sarcoma in rats. The data (table 4) show that no effect of the tumor on the phosphatase activity of the tissues of tumor-bearing animals could be observed.

THE EFFECT OF ADDED MAGNESIUM IONS

It has been pointed out that magnesium ions cause an activation of concentrated preparation of tissue phosphatase (*1*).[4] Although it would hardly be expected that moderately small amounts of added

[4] *Bodansky* (13) *showed that amino acids serve as activators for phosphatase, and Hove, Elvejhem, and Hart* (15) *showed that a combination of zinc ions and alpha amino acids strongly activate tissue phosphatase. The fact that influences other than the presence of metallic salts and protein split-products play a role in the activation of phosphatase is illustrated in the work of Maver and Voegtlin* (16). *The latter investigators showed that enhanced nuclease activity (increased inorganic phosphate) at the pH optima was obtained on changing the digestion conditions from aerobic to anaerobic.*

TABLE 4.—*The acid and alkaline phosphatase activity of certain tissues of tumor-bearing animals*
[5 cc. M/200 disodium phenylphosphate incubated at pH 4.6 and pH 9.5 for 1 hour at 37.5° with 0.5 cc. extract solution]

Source of material	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Milligrams per cubic centimeter	*Per cent*		*Per cent*	
Tissues of Osborne-Mendel rats carrying transplanted hepatic tumor 31:[1]					
Liver	4.2	90	21	12	3
	.84	17	20	2	2
	.42	8	19	1	2
	.08	1	13	0	.....
Average	.....	.....	18	.....	2
Kidney	.42	42	100	100	.....
	.21	20	95	100	.....
	.04	5	125	70	1,750
	.008	0	.....	13	1,625
	.004	0	.....	7	1,750
Average	.....	.....	107	.....	1,708
Spleen	2.0	80	40	55	27
	.4	15	38	11	27
	.2	7	35	6	30
	.04	1	25	1	25
Average	.....	.....	35	.....	27
Muscle	2.0	10	5	4	2
	.4	2	5	1	2
	.2	0	.....	0	.....
	.04	0	.....	0	.....
Average	.....	.....	5	.....	2
Skin	1.0	9	9	5	5
	.2	2	10	0	.....
	.1	0	.....	0	.....
	.02	0	.....	0	.....
Average	.....	.....	9	.....	5
Blood serum	9.8	4	.4	86	8
	1.96	1	.5	17	8
	.98	0	.....	9	9
	.19	0	.....	2	10
Average	.....	.....	.4	.....	9
Tissues of Buffalo rats carrying transplanted Jensen sarcoma:[1]					
Liver	3.6	88	24	10	3
	.72	18	25	2	2
	.36	9	25	1	3
	.07	2	28	0	.....
Average	.....	.....	25	.....	3
Kidney	.42	40	95	100	.....
	.21	20	95	100	.....
	.04	4	100	60	1,500
	.008	0	.....	11	1,375
	.004	0	.....	6	1,500
Average	.....	.....	97	.....	1,458
Spleen	2.2	91	41	60	27
	.44	17	38	12	27
	.22	8	37	6	27
	.04	1	25	1	25
Average	.....	.....	35	.....	26

Source of material	Concentration of *N* in extract	Hydrolysis at pH 4.6	*Q* (acid)	Hydrolysis at pH 9.5	*Q* (alkaline)
	Milligrams per cubic centimeter	*Per cent*		*Per cent*	
Tissues of Buffalo rats carrying transplanted Jensen sarcoma—Con.					
Muscle	2.0	10	5	4	2
	.4	2	5	1	2
	.2	0	.....	0	.....
	.04	0	.....	0	.....
Average	.....	.....	5	.....	2
Skin	.05	7	14	3	6
	.1	1	10	0	.....
	.05	0	.....	0	.....
	.01	0	.....	0	.....
Average	.....	.....	12	.....	6
Blood serum	9.8	4	.4	90	9
	1.96	1	.5	16	8
	.98	0	.....	7	8
	.19	0	.....	1	6
Average	.....	.....	.4	.....	8
Tissues of I × dilute brown mice carrying transplanted hepatic tumor 1:[1]					
Liver	3.6	40	11	15	4
	.72	8	11	3	4
	.36	4	11	1	3
	.07	1	14	0	.....
Average	.....	.....	12	.....	4
Kidney	.44	8	18	100	.....
	.22	4	18	100	.....
	.04a	1	22	40	909
	.008	0	.....	9	1,125
	.004	0	.....	4	1,000
Average	.....	.....	19	.....	1,011
Spleen	2.2	100	.....	32	14
	.44	36	82	6	14
	.22	19	86	3	14
	.04	4	100	0	.....
Average	.....	.....	89	.....	14
Muscle	2.0	36	18	4	2
	.4	7	17	1	2
	.2	3	15	0	.....
	.04	0	.....	0	.....
Average	.....	.....	17	.....	2
Skin	.5	10	20	4	8
	.1	2	20	1	10
	.05	0	.....	0	.....
	.01	0	.....	0	.....
Average	.....	.....	20	.....	9

[1] The ages of the transplanted tumors are as follows: mouse hepatic, 3 weeks; rat hepatic, 4 weeks; Jensen sarcoma, 3 weeks. The ages given refer to the time elapsed since implantation of the respective tumors.

magnesium ions would produce a significant effect on tissue extracts in which the normal complement of enzyme activators presumably are present, nevertheless it was considered necessary to test the effect on the enzymatic activity of certain tissue extracts by added magnesium. The tissues selected were normal rat liver and the transplanted rat hepatoma 31. Both acid and alkaline phosphatase activity in the tumor were found to be considerably greater than in normal liver. Addition of magnesium chloride to extracts of either tissue (table 5) produced no change in the phosphatase activity of the tissue concerned.

TABLE 5.—*The effect of added magnesium on the phosphatase activity of normal rat liver and the transplanted hepatic tumor of the rat*

[5 cc. M/200 disodium phenylphosphate incubated at pH 4.6 and pH 9.5 for 1 hour at 37.5° with 0.5 cc. extract solution. To the test determinations 0.05 cc. of 0.005 M magnesium chloride was added; to the controls 0.05 cc. of water was added]

Tissue	Concentration of N in extract	Hydrolysis at pH 4.6		Q (acid)		Hydrolysis at pH 9.5		Q (alkaline)	
		Test	Control	Test	Control	Test	Control	Test	Control
	Milligrams per cubic centimeter	*Percent*	*Percent*			*Percent*	*Percent*		
Normal rat liver	3.6	86	86	23	23	10	11	3	3
	.7	17	17	23	23	2	2	3	3
	.36	9	9	25	25	1	1	3	3
	.07	2	2	28	28	0	0	.....	.....
Average	.....	.....	.....	25	25	.....	.....	3	3
Transplanted hepatoma 31 in Osborne-Mendel rats	.2	11	11	55	55	100	100	.....	.....
	.04	2	2	50	50	25	25	625	625
	.008	0	0	.....	.....	5	5	625	625
	.004	0	0	.....	.....	2	2	500	500
Average	.....	.....	.....	52	52	.....	.....	583	583

DISCUSSION

In evaluating the data, it is of interest first to segregate the various tissues according to the relative activity of acid *Q* (acid) and alkaline *Q* (alkaline) phosphatase. In the following columns the tissues are separated into three groups:

Q (acid) > *Q (alkaline)*	*Q (acid)* = *Q (alkaline)*	*Q (acid)* < *Q (alkaline)*
Lymph nodes.	Bone marrow.	Intestinal mucosa.
Liver.	Mouse lung.	Kidney.
Muscle.	Spontaneous mammary tumors in mice.	Blood serum.
Spleen.	Transplanted lymphoma 72942 in mice.	Jensen rat sarcoma.
Skin.		Transplanted rat hepatoma 31.
Hyperplastic mammary tissue in mice.		
Gastric mucosa in mice.		
Transplanted intestinal tumors in mice.		
Transplanted stomach tumors in mice.		
Transplanted lung tumors in mice.		
Transplanted hepatic tumors in mice.		
Transplanted malignant endothelioma in mice.		
Transplanted malignant melanoma in mice.		
Transplanted sarcoma 37 in mice.		
Transplanted sarcoma CR 180 in mice.		
Primary induced tumor (benzpyrene) in mice.		

The fact has long been known that the acid phosphatase in liver and spleen is more active than the alkaline phosphatase of these organs and that the reverse holds true in the case of the intestinal mucosa, kidney, and blood serum (*1*, *17*) However, the substrate hitherto employed for the phosphatase of these tissues was β-glycerophosphate, while in the present work, monophenylphosphate was employed. The problem of whether the phosphatases of tissues such as the spleen and the kidney are identical has already been investigated (*1*, *17*, *18*). For the purpose of the present paper this problem is largely of academic interest, but it may be pointed out that in the absence of sufficiently purified preparations of the enzyme a satisfactory answer to the problem is not as yet possible. The same may be said of attempts to distinguish the acid and alkaline phosphatase of any one tissue, for these attempts, which have largely been based on selective inhibition experiments (with fluoride, etc.), were necessarily performed on crude materials at widely differing pH levels.

The alkaline phosphatase activity of the induced and all of the transplanted mouse tumors studied is either zero or little greater than zero. The acid phosphatase activity of all of the mouse tumors, induced, spontaneous, and transplanted, is approximately of the same order, the values of Q (acid) ranging between 6 and 23. With the exception of the spleen, the values of Q (acid) for normal mouse tissues fall within the latter range. The alkaline phosphatase activity of the transplanted mouse tumors is almost nonexistent, that of the transplanted rat tumors is very high. The only mouse tumor with high alkaline phosphatase activity is the spontaneous mammary carcinoma. In general, the phosphatase activity of rat tissues, both normal and cancerous, is considerably greater than that of comparable tissues in the mouse. This condition is quite the opposite of that found in all other enzymes previously studied (*5*).

Hepatic Tissues

When the relative activity of acid and alkaline phosphatase in mouse liver and mouse transplanted hepatomas is compared with that in rat liver and the transplanted hepatoma 31, the distribution of the enzymes is found to be different for each species. In the normal mouse liver and in the transplanted hepatic tumor in I × dilute brown and C3H mice, Q (acid) is much greater than Q (alkaline). As a matter of fact, Q (alkaline) in each of the transplanted mouse hepatic tumors is zero, or nearly zero, and thus much less than that of normal mouse liver. Q (acid) in each of the mouse tumors is either equal to or somewhat greater than that of normal mouse liver. In contrast, Q (alkaline) is very much greater than Q (acid) in the transplanted rat hepatic tumor, and both Q (alkaline) and Q (acid) for the tumor are much greater than the corresponding values for normal rat liver. As already pointed out, the value for the alkaline phosphatase activity of the rat hepatic tumor is among the highest yet observed for any of the animal tissues studied. The reason for the difference in the distribution of acid and alkaline phosphatase in the transplanted hepatic tumors of the mouse on the one hand, and of the rat on the other hand, is not known. The virtual disappearance of alkaline phosphatase activity in the mouse hepatic tumors and the extraordinarily high value for alkaline phosphatase activity in the rat hepatic tumor illustrate vividly the fact that the chemical characterization of a tumor must take into account the species from which the tumor is derived. Furthermore, it is apparent that in such a characterization

the activity of not one, but several enzyme systems must be considered. The transplanted hepatic tumors in rats and mice in comparison with normal liver have very low arginase and catalase activity and slightly lower or equal xanthine dehydrogenase, amylase, and thymonucleodepolymerase activity (*2, 4, 5*). This partial characterization is nearly the same for the hepatic tumor in both species. How different a characterization for each species based on phosphatase activity must be is illustrated by the data referred to above.

Like the hepatic tumors in the mouse, the alkaline phosphatase activity of fetal rabbit liver is zero or nearly so. On the other hand, the acid phosphatase activity of the fetal liver is somewhat greater than that of normal adult rabbit liver. Essentially, however, the distribution of acid and alkaline phosphatase in fetal liver is the same as in adult liver, for in both kinds of tissue Q (acid) is much greater than Q (alkaline).

Intestinal and Gastric Tissues

The alkaline phosphatase activity of normal mouse intestinal mucosa is the highest found for any of the animal tissues studied. In the intestinal adenocarcinoma this activity drops to about 0.1 percent of the value in the normal tissue. This represents a tremendous drop in activity and reflects the difference between a functioning and at least a nonspecific functioning tissue. The acid phosphatase activity of the intestinal adenocarcinoma is about half that of the normal intestine. The distribution of acid and alkaline phosphatase is different in the normal and in the tumor tissue, for in the former Q (acid)$<Q$ (alkaline) whereas in the latter Q (acid)$>Q$ (alkaline).

In normal mouse gastric mucosa as well as in the transplanted adenocarcinoma of the mouse stomach Q (acid)$>Q$ (alkaline). Both Q (acid) and Q (alkaline) are lower in the tumor than in the normal tissue. As in the case of the transplanted hepatic tumors of the mouse, the alkaline phosphatase of the mouse transplanted stomach adenocarcinoma is zero or nearly so.

Mammary Tissues

The spontaneous mammary tumors in both C3H and A strains of mice appear to have equal acid and alkaline phosphatase activity, and, moreover, in each tumor the acid phosphatase is equal to the alkaline phosphatase activity. In hyperplastic mammary tissue induced by stilbestrol, the acid phosphatase is about twice as active as the alkaline phosphatase. A comparison of the hyperplastic with cancerous mammary tissue reveals that the acid phosphatase activity is nearly the same for both kinds of tissue but that the alkaline phosphatase activity is twice as great in the tumor.

Lymphatic Tissues

The acid phosphatase activity of normal mouse lymph nodes is greater than that of either bone marrow or lymphoma 72942. The alkaline phosphatase activity of the nodes is of the same order as that of the lymphoma and is less than that of marrow. In marrow and in the lymphoma the acid phosphatase activity is equal to the alkaline phosphatase activity.

Tissues of Normal and Tumor-Bearing Animals

Comparison of the phosphatase activity, both acid and alkaline, of the liver, kidney, spleen, skin, muscle, and sera of animals bearing transplanted tumors and that of comparable tissues and sera in normal animals reveals no significant differences. This finding places phosphatase in the same category of enzymes as xanthine dehydrogenase, amylase, and thymonucleodepolymerase, the activity of

which in the tissues of tumor-bearing animals appears to be unchanged from that characteristic of normal animals, and in marked contrast with catalase and arginase the activity of which is much lower in tumor-bearing than in normal animals (*2*, *6*, *7*). It should be pointed out that the present results on phosphatase are at variance with the results reported by Köhler (*8*).

The phosphatase activity of human serum in pathologic conditions has been intensively investigated (*19–22*), and significant increases in the activity of this enzyme have been reported particularly in the cases of bone diseases and of prostatic malignancy. Gutman and Gutman (*21*) and later Huggins and Hodges (*22*) found that the acid phosphatase of the serum of patients with carcinoma of the prostate is considerably more than that of normal serum. In collaboration with Dr. Charles Lenhoff, we have recently confirmed this finding. The failure to find any difference from the normal phosphatase value in the sera of rats bearing the transplanted hepatic and the Jensen tumors is not altogether surprising since there is no reason why the sera of different species should behave in the same manner in cases of malignancy of the animals concerned. The very high acid phosphatase activity of the serum of individuals with metastasizing prostatic carcinoma has been related to the very high acid phosphatase activity of the primary tumor. The transplanted hepatic tumor 31 in the rat has an unusually high alkaline phosphatase activity, yet the sera of animals bearing this tumor do not show enhanced alkaline phosphatase activity. The sera of human beings with prostatic carcinoma and of rats with transplanted hepatoma are little comparable, and the difference noted may be explained on any number of obvious grounds.

SUMMARY

The acid and alkaline phosphatase activity of a number of normal and cancerous tissues and sera were investigated. These tissues included the lymph nodes and marrow of mice and the liver, kidney, spleen, skin, and muscle of normal and tumor-bearing rats and mice; the sera of normal and tumor-bearing rats; fetal and adult rabbit liver; the Jensen sarcoma and the transplanted hepatoma 31 in rats; spontaneous mammary tumors in two strains of mice; primary benzpyrene-induced tumors in mice; intestinal, lymphatic, gastric, pulmonary, hepatic, endothelial, and melanotic transplanted tumors in mice; and sarcomas 37 and CR 180.

Varying ratios of enzyme to substrate (phenylphosphate) for all of the tissues studied revealed that a linear relation existed between the phosphatase activity and the concentration of the tissue extracts in milligrams of total N per cubic centimeter at least over the range of concentrations employed. The enzyme followed the course of a monomolecular reaction as revealed by kinetic studies.

When the relative acid and alkaline phosphatase of each tissue is considered, it is found that the tissues may be divided into three categories: (1) Tissues in which the acid phosphatase is more active than the alkaline phosphatase, namely, liver, spleen, nodes, muscle, skin, gastric mucosa, hyperplastic mammary tissue, the primary benzpyrene-induced mouse tumor, and all of the transplanted tumors in mice reported in this paper; (2) tissues in which the acid phosphatase is nearly equal in activity to the alkaline phosphatase, namely, normal mouse lung, bone marrow, lymphoma 72942, and spontaneous mammary tumors in mice; (3) tissues in which the acid phosphatase is less than the alkaline phosphatase, namely, intestinal mucosa, kidney, the Jensen rat

sarcoma, and the transplanted hepatoma 31 in rats. Blood serum falls into the last category, inasmuch as the acid phosphatase is less than the alkaline.

With the exception of the spontaneous mammary tumors and the lymphoma, all of the mouse tumors studied possess very little or no alkaline phosphatase activity. The acid phosphatase activities of all of the mouse tumors, on the other hand, are high and are of approximately the same order of activity. The rat tumors possess very high alkaline phosphatase activity, in particular the transplanted hepatic tumor, the alkaline phosphatase activity of which is extraordinarily great. The acid phosphatase activity of the rat tumors, as well as of normal rat tissues, is considerably higher than that of mouse tumors and normal mouse tissues.

The study of pairs of comparable tumors and normal control tissues showed that: (1) For mouse hepatic tissues the acid phosphatase is practically the same in normal liver and in the transplanted hepatic tumors in I × dilute brown and in C3H mice, but the alkaline phosphatase is very much less in the tumors than in liver, and for rat hepatic tissues both acid and alkaline phosphatase activity is much greater in the transplanted tumor than in normal liver, the increase being very much greater for the alkaline than for the acid phosphatase; (2) for mouse mammary tissue the acid phosphatase is nearly the same for hyperplastic mammary tissue and for the spontaneous tumors, but the alkaline phosphatase is much higher in the latter than in the former tissue; (3) for mouse gastric and intestinal tissue both acid and alkaline phosphatase activity is much lower in the transplanted adenocarcinomas than in the respective normal mucosas, the decrease being far greater for the alkaline than for the acid phosphatase; and (4) for mouse lymphatic tissue, the transplanted lymphoma is lower in acid phosphatase activity than either lymph nodes or bone marrow whereas the alkaline phosphatase activity of the tumor is about the same order as that of the nodes and less than that of the marrow. Comparison of the activity of fetal and adult rabbit liver showed that the former tissue has a greater acid phosphatase and a much weaker alkaline phosphatase activity than the latter. The alkaline phosphatase activity of the fetal rabbit liver appears to be zero or nearly zero. In both kinds of tissue, however, the acid phosphatase is much greater than the alkaline phosphatase activity.

Studies of the phosphatase activity of the liver, kidney, spleen, skin, muscle, and sera of mice and rats bearing transplanted tumors revealed no significant difference from the activity of the same tissues and sera in normal animals.

The unusually high alkaline phosphatase activity of the transplanted hepatoma 31 in rats is emphasized. The activity of this tumor at 7, 14, 21, 29, 35, and 56 days following implantation was practically the same at each stage of growth. Addition of magnesium ions to extracts of this tumor and of normal rat liver produced no change in the respective activity of these from that of the untreated extracts.

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Titration of the Liver Catalase Activity of Normal and of Tumor-Bearing Rats and Mice

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It has been observed that the liver catalase activity of rats and of mice bearing tumors is very much lower than that of normal animals of these species (*1–4*). This phenomenon has been studied in a wide variety of tumor-bearing animals, and the following general statements may be made at this time: (1) The effect on the liver catalase is most marked in animals bearing the most rapidly growing tumors; (2) the effect on the liver catalase is progressive with the growth of the tumor, approaching a limiting value which appears to be dependent on the tumor concerned; and (3) the effect is reversible in both rats and mice, for removal of the tumor results in a rapid restoration of the liver catalase activity to the normal level.

The only exceptions to the finding of a lowered liver catalase activity in tumor-bearing animals have been observed in (1) C3H and A strain mice bearing the very slowly growing transplanted hepatoma and intestinal adenocarcinoma, respectively, (2) C3H and dilute brown strain mice bearing the relatively slowly growing spontaneous mammary adenocarcinoma and melanoma, respectively, in the early stages of tumor growth, and (3) C57 Black mice bearing the rapidly growing transplanted sarcoma 37. The last-mentioned exception is an illustration of the role which strain differences in mice may play, for the same tumor in other strains produces a profound effect in lowering the liver catalase activity.

The experimental technique used in determining the liver catalase activity depended primarily on two factors: (1) Since the studies mentioned (*1–4*) were essentially comparative, it was considered necessary to make the activity determinations at equal concentrations of total N per cubic centimeter of the liver extracts of the normal and of the tumor-bearing animals; and (2) the rate of oxygen evolved by the interaction of hydrogen peroxide and the liver extract was measured, and the linear portion of the rate curve used to estimate the relative activity. The latter is a volumetric method, and its advantages consist in direct readings of activity and in the extremely small period of contact of the enzyme with the substrate. This method has been shown to be capable of excellent reproducibility of the data (*2*). The only obvious disadvantage when using a single size of apparatus consists in the inability to measure activities conveniently over a wide range of varying enzyme and substrate concentrations. The latter studies are more conveniently pursued by a titration method. In this method the rate of disappearance of the hydrogen peroxide is followed by the use of either permanganate or hydriodic acid (*5*). In order, therefore, to compare the liver catalase activity of normal and of tumor-

bearing animals over a wide range of enzyme and substrate concentrations the titration method was resorted to, and the results obtained by using this method are described in the present paper.

Experimental Procedure

The volumetric method, by which the rate of oxygen evolution is measured, was used in the previous studies. The actual technique used was modeled on that of White and Winternitz (*6*). Since the technique was not described in the earlier papers (*1–4*), it seems advisable to give a brief resume of the method. The phosphate buffer and the liver extract were placed in the reaction container, a 100-cc. wide-mouthed, flat-bottomed, glass bottle. The hydrogen peroxide solution was placed in a tiny flat-bottomed glass flask of slightly more than 1-cc. capacity, and the flask then carefully placed in the glass bottle containing the mixture of buffer and enzyme solution. The bottle was covered with a one-hole rubber stopper, through which was passed a glass tube connected by flexible rubber tubing to a glass side-arm sealed near the top of a 50-cc. burette. The top of the burette was closed with a rubber stopper containing a one-way stopcock, and the bottom of the burette was attached, by means of rubber tubing, to a leveling bulb filled with water. All joints were treated with shellac. The glass bottle holding the enzyme-buffer mixture and the flask containing the peroxide was fastened on a horizontal shaker, the leveling bulb adjusted, and the whole apparatus closed. When the shaker was started, the flask containing the peroxide was tipped over, and the oxygen that was evolved was measured along the burette as a function of the time elapsed since shaking was begun. The time was observed by means of a stop watch. Under the conditions described (*1–4*), the time required for 20 cc. of oxygen to be evolved was about 12 seconds for the liver extract of normal rats at 0.6 mg. N per cubic centimeter, and approximately 210 seconds for the liver extract of tumor-bearing rats. For both kinds of extract the rate of oxygen evolution was nearly linear up to the evolution of 20 cc., and the slopes of the rate curves were used for the data (*2*). The results on the livers of normal and of tumor-bearing mice were similarly obtained. The variation in activity between individual animals was ± 20 percent.

The titration method used is a modification of the Jolles-Stern technique described by Sumner (*7*). This consists in making up a solution of hydrogen peroxide of low normality in 0.015 M phosphate buffer and incubating at 0° C. with liver extract. Fifty cubic centimeters of the buffer-substrate solution at 0° is treated with 1.0 cc. of the suitably diluted liver extract, and at intervals 5.0 cc. of this mixture is removed and added to 10 cc. of 10 percent sulfuric acid. To this are added 10 cc. of 10 percent potassium iodide solution and 1 cc. of ammonium molybdate solution. The liberated iodine is then titrated with 0.05 N thiosulfate and starch. Suitable controls are simultaneously run, and from these the normality of the peroxide substrate at every time interval can be calculated. The iodine titrated is, of course, inversely proportional to the amount of hydrogen peroxide decomposed by the catalase. The results, given in terms of percent of splitting, are substituted in the expression for the monomolecular reaction, and the expression k is calculated:

$$k=\frac{2.3}{t}\log\frac{100}{100-x}$$

where t is the time in minutes and x is the percent of hydrogen peroxide decomposed

in the time designated. Under the conditions employed, k is not a constant quantity but decreases in value with increasing time of incubation. No attempt, therefore, was made to estimate its absolute value at zero time although an extrapolation is possible (7). Instead, the ratios of k are given for the livers of normal and of tumor-bearing animals, respectively, under identical conditions, i. e., period of incubation and concentration of the liver extracts in milligrams of N per cubic centimeter.

The tissues were the livers of twelve normal rats and mice, the livers of twelve rats bearing transplanted Jensen sarcomas and transplanted hepatic tumors, and the livers of six dilute brown mice bearing the transplanted sarcoma 37. Individual variation was ± 20 percent in activity. They were prepared in exactly the same way as that described earlier (*1–4*). Simultaneously, the catalase activity of these tissues was determined by the volumetric method and the same results obtained as before (*2, 4*).

The first investigations were performed at several concentrations of the liver extracts and at a single concentration of the substrate. The averaged results are given in table 1.[1]

Table 1 reveals the striking difference between the liver catalase activity of normal and of tumor-bearing rats and mice. The data thus confirm the earlier findings by the volumetric method (*1–4*). The larger values of k for the mouse livers than for the rat livers are also consistent with the earlier findings.

TABLE 1.—*The titrimetric determination of the liver catalase activity of normal and of tumor-bearing animals.*

50 cc. of 0.0093 N H_2O_2 in 0.015M phosphate buffer at pH 6.9 incubated at 0° C. with 1.0 cc. liver extract; aliquots of 5.0 cc. removed at intervals and treated successively with H_2SO_4, KI, and ammonium molybdate; finally the liberated I_2 was titrated with 0.05N thiosulfate.

Tissue	Concentration of N in extract	Time of incubation	Splitting of H_2O_2	k
	Milligrams per cubic centimeter	*Minutes*	*Percent*	$\times 10^4$
Normal rat livers	0.2	5	75	2,769
		10	93	2,657
	.1	5	54	1,555
		10	76	1,425
		15	84	1,280
	.05	5	23	524
		10	35	430
		15	43	366
		20	52	360
		25	58	340
Livers of rats bearing transplanted hepatic tumor 31 for 8 weeks [1] (weight of tumors 15–40 gm.)	.2	5	40	1,016
		10	60	915
	.1	5	30	708
		10	38	476
		15	43	366
	.05	5	9	189
		10	14	150
		15	18	129
		20	21	112
		25	24	107
Livers of rats bearing transplanted Jensen sarcoma for 3 weeks [1] (weight of tumors 20–30 gm.)	.2	5	31	740
		10	50	692
	.1	5	18	395
		10	28	329
		15	35	281
	.05	5	7	142
		10	11	117
		15	14	98
		20	17	89
		25	20	86
Normal dilute brown mouse liver	.2	5	97	7,001
	.1	5	77	2,935
		10	93	2,657
	.05	5	43	1,122
		10	66	1,076
		15	76	929
		20	83	846
Livers of dilute brown mice bearing transplanted sarcoma 37 for 10 days [1] (weight of tumors 5–7 gm.)	.2	5	55	1,592
	.1	5	30	708
		10	43	561
	.05	5	11	230
		10	20	223
		15	26	195
		20	31	177

[1] The age of the tumor refers to the time elapsed since implantation.

The ratios of $k:k$ under identical conditions are given in table 2.

Table 2 shows that the livers of rats bearing the transplanted Jensen sarcoma have about one-fourth, and the livers of rats bearing the transplanted hepatic tumor about one-third the catalase ac-

[1] *It will be noted that the approximate weight of the tumors is given in table 1. The reason for this is that we have found that the effect on the liver catalase of animals bearing the transplanted hepatic tumor, S 37, or the Jensen sarcoma depends on the size of the tumor. Thus, the liver catalase of a rat bearing a Jensen sarcoma for 3 weeks which weighed only 2–3 gm. at the end of that period was barely lower than that of normal liver.*

TABLE 2.—*Ratio of* k (*normal animal livers*) *to* k (*livers of tumor-bearing animals*)

[The concentration of H_2O_2 used as substrate was 0.0093 N in 0.015 M phosphate buffer at pH 6.9]

Tissue pairs	Concentration of N in extract	Time of incubation	Ratio $k:k$
	Milligrams per cubic centimeter	*Minutes*	
Normal rat livers and livers of rats bearing transplanted hepatic tumor 31 for 8 weeks	0.2	5	2.7
		10	2.8
	.1	5	2.2
		10	2.9
		15	3.5
	.05	5	2.7
		10	2.8
		15	2.9
		20	3.2
		25	3.2
Average			2.9
Normal rat livers and livers of rats bearing transplanted Jensen sarcomas for 3 weeks	.2	5	3.7
		10	3.8
	.1	5	3.8
		10	4.3
		15	4.2
	.05	5	3.5
		10	3.6
		15	3.7
		20	4.0
		25	3.9
Average			3.8
Livers of normal dilute brown mice and of dilute brown mice bearing transplanted sarcoma 37 for 10 days	.2	5	4.5
	.1	5	4.2
		10	4.8
	.05	5	4.8
		10	4.8
		15	4.7
		20	4.8
Average			4.7

tivity of normal rat liver. The livers of mice bearing transplanted sarcoma 37 have about one-fifth the activity of normal mouse liver. These ratios are somewhat greater than those found by the volumetric method. The ratio $k:k$ for each pair of tissues is nearly constant over a fourfold dilution of the respective liver extracts.

Investigations were also made on mixtures in which the amounts of substrate and of enzyme were substantially increased. The livers of normal rats and of rats bearing the Jensen sarcoma were used. The data are given in table 3.

The data in table 3 reveal that substantially the same ratio of $k:k$ for the normal and tumor-bearing livers is obtained over a considerable range of enzyme and substrate concentrations. The value for this ratio is somewhat higher than that given in table 2 for lower concentrations of the substrate. This relatively

TABLE 3.—*The effect of varying the concentration of substrate and of enzyme in extracts of liver of normal rats and of rats bearing the Jensen sarcoma* [1]

[50 cc. of H_2O_2 solutions in 0.015M phosphate buffer at pH 6.9 incubated at 0° C. with 1.0 cc. liver extract; aliquots of 5.0 cc. removed at intervals and treated successively with H_2SO_4, KI, and ammonium molybdate; finally the liberated I_2 was titrated with 0.05N thiosulfate.]

Rat tissue	Concentration of H_2O_2	Concentration of N in extract	Incubation period	Splitting of H_2O_2	k	Ratio $k:k$
	N	*Milligrams per cubic centimeter*	*Minutes*	*Percent*	$\times 10^4$	
Normal liver	0.0165	0.2	10	89	2,203	4.2
Livers of rats bearing Jensen sarcoma				41	527	
Normal liver			20	95	1,431	4.6
Livers of rats bearing Jensen sarcoma				48	312	
Normal liver		.1	5	22	496	4.0
Livers of rats bearing Jensen sarcoma				6	124	
Normal liver			10	32	384	4.7
Livers of rats bearing Jensen sarcoma				8	82	
Normal liver			15	40	331	4.4
Livers of rats bearing Jensen sarcoma				11	75	
Normal liver	.1475	1.2	10	18	198	4.0
Livers of rats bearing Jensen sarcoma				5	50	
Normal liver			20	28	156	4.6
Livers of rats bearing Jensen sarcoma				7	34	
Normal liver			30	35	130	4.3
Livers of rats bearing Jensen sarcoma				9	30	
Normal liver		.6	15	35	281	4.5
Livers of rats bearing Jensen sarcoma				9	62	
Normal liver			30	45	190	4.2
Livers of rats bearing Jensen sarcoma				14	45	
Normal liver			45	55	173	4.2
Livers of rats bearing Jensen sarcoma				17	41	

[1] The age of the Jensen sarcoma, i. e., time elapsed since implantation, was 3 weeks. The average weight of the tumor was 30 gm.

minor discrepancy is probably due to experimental error, since the removal of aliquots saturated with oxygen from digests in which a large amount of peroxide is decomposed is attended with some difficulty. The aliquot taken from the normal liver digests will probably be slightly smaller in actual liquid volume than that from the digests of the livers of tumor-bearing animals, and hence the values of k for the former will tend to be somewhat high.

A further point of interest lies in the respective values of k for the normal liver and liver from the tumor-bearing animal at the higher concentration of peroxide used (table 3). The value of k is lower for the higher concentration of N in the extracts used, a phenomenon not encountered where lower concentrations of both peroxide and extract are used (tables 1 and 3).

The ratios of k, obtained under identical conditions, are equivalent to ratios of *Kat. f.*, the conventional and most widely used method of expressing catalase activity (*5*).

DISCUSSION

The data in tables 1, 2, and 3, obtained by the use of a titration method, indicate that the liver catalase activity of tumor-bearing animals is considerably lower than that of normal animals. This result confirms the data obtained earlier by the use of the volumetric method for measuring catalase activity (*1–4*). By the use of the latter method, with the same tissues, the ratio of the catalase activity of tumor-bearing to that of normal rats was approximately 1:15. The ratio of the catalase activity of mice bearing sarcoma 37 to that of normal mice was about 1:20. The same ratios obtained by the titration method are, respectively, 1:3–4 and 1:5. The reason for the discrepancy is not apparent, although results obtained by the two methods are not strictly comparable in view of the considerably different conditions employed in each.

The evolution of oxygen in the volumetric method is almost explosive in quality, and the reaction occurs within an extremely small period. The digestion in the titration method takes a much longer period, while it proceeds at a lower temperature. It is possible that because of the much shorter period of reaction between enzyme and substrate in the volumetric method that differences between the liver extracts are demonstrated more sharply than in the titration method. In any event, no matter what explanation may be advanced to explain the differences in ratios found between the two methods, both methods reveal the very much lower catalase activity in the livers of tumor-bearing animals.

SUMMARY

Supplementing previous determinations of the liver catalase activity of normal and of tumor-bearing animals by a volumetric method, a titration method was employed for the determination of the liver catalase activity of normal rats and of rats bearing the transplanted hepatic tumor 31 and the transplanted Jensen sarcoma, as well as of normal mice and of mice bearing the transplanted sarcoma 37.

The data for the comparison of the liver catalase activity of the normal and the tumor-bearing animals were given as ratios, under identical conditions, of the values of k, the monomolecular reaction constant, for each tissue. The liver catalase activity ratio of tumor-bearing to normal rats was 1:3–4, and the ratio of tumor-bearing to normal mice about 1:5. These results are consistent with those obtained on the same tissues by the volu-

metric method, although the differences found by the latter method are greater. By either method it is obvious that the liver catalase activity of tumor-bearing animals is considerably less than that of normal animals.

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